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The Polynuclear Aromatic Hydrocarbons in Cigarette-Smoke Condensate. II.[1,2]

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Summary

With the use of methods previously described, the following polynuclear aromatic hydrocarbons, in addition to the 7 previously reported, were detected in cigarette-smoke condensate: chrysene, perylene, benzo[*mno*]-fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, 1,2-benzfluorene, dibenz[*a*,*b*]anthracene, a methylbenzo[*a*]pyrene, benzo[*c*]-phenanthrene, an alkylfluoranthene, and an alkylchrysene. In addition, one heterocyclic compound, 1,8,9-perinaphthoxanthene, was identified. The concentrations of some of these compounds in cigarette-smoke condensate were determined.—J. Nat. Cancer Inst. 21: 623–630, 1958.

In a previous communication (*1*) we described the identification of a number of condensed aromatic hydrocarbons from cigarette-smoke condensate. The successful results obtained were due mainly to the use of paper chromatography combined with spectral methods of identification. Microgram quantities of the hydrocarbons were obtained in relatively pure form and some hydrocarbons were determined quantitatively. In this report we describe the continuation of the work in an attempt to account for the known carcinogenic activity of cigarette tars to mouse skin (*2*, *3*). The identification of 12 additional aromatic compounds, including 11 hydrocarbons and 1 heterocyclic aromatic compound, is presented in this paper. Additional paper chromatographic methods of aromatic hydrocarbons were examined in the course of this work.

Experimental

Identification of aromatics.—The experimental procedure and fractions used were the same as those previously described (*1*). The compounds

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identified in this study and the fractions from which they were obtained are given in table 1. In some instances it was possible to calculate concentrations from bands in the ultraviolet absorption spectra. All the hydrocarbons required 3 to 4 rechromatographies on paper for purification. The fractions were rechromatographed until there was no further change in the appearance of their ultraviolet absorption spectra and no further change in paper chromatographic behavior. The compounds were identified as before by comparison of R_F values, ultraviolet absorption spectra, fluorescence excitation spectra, and fluorescence emission spectra with those of pure authentic materials. Agreement in all criteria was required for assignment of identity.

TABLE 1.—*Origin of aromatic compounds*

Compounds identified	Origin*	
	Fraction number	Subfraction numbers
Alkylfluoranthene	III	104 to 107
Chrysene	IV	1 to 6
Alkylchrysene	III	104 to 107
Methylbenzo[*a*]pyrene	IV	1 to 6
Perylene	IV	1 to 6
Benzo[*mno*]fluoranthene	III	126, 127, 128
Benzo[*k*]fluoranthene (8,9-Benzfluoranthene)	IV	1 to 6
Benzo[*j*]fluoranthene (7,8-Benzfluoranthene)	IV	1 to 6
1,2-Benzfluorene	III	129
1,8,9-Perinaphthoxanthene	III	129
Dibenz[*a*,*h*]anthracene	IV	1 to 6
Benzo[*c*]phenanthrene	III	99

*Fractions III and IV are the petroleum ether and benzene eluates, respectively, from the chromatography of the neutral fraction of cigarette-smoke condensate on acid-washed alumina. The subfractions were obtained by rechromatography of fractions III and IV on activated alumina. The details of the fractionation were given in our previous communication (*1*).

Chromatography of aromatic hydrocarbons on acetate paper.—Whatman #1 paper was acetylated with a mixture of toluene and acetic anhydride (1 : 3) containing a few drops of concentrated sulfuric acid. This treatment was carried out at room temperature for 36 hours. The solvent was then decanted and the paper allowed to stand under methanol for 24 hours and then under water for 24 hours. The paper was finally dried at 120° C. for 1 hour. The solvent system used was that described by Bergmann (*4*) and consisted of a mixture of toluene, methanol, and water (1 : 10 : 2). Chromatographies were run as described before (*1*). The R_F values obtained for a number of hydrocarbons are given in table 2.

Discussion

A complete list of all the hydrocarbons identified to date, together with concentrations and carcinogenic activity, is given in table 3.

The fractionation procedure developed and used in this study was designed primarily for the isolation and identification of aromatic hydro-

TABLE 2.—*Average* R_F *values with acetate paper*

Compound	R_F on acetate paper	R_F on untreated Whatman #1 paper (*1*)
Fluoranthene	0. 170	0. 69
Pyrene	0. 130	0. 71
Perylene	0. 079	0. 46
5-Methylbenzo[*a*]pyrene	0. 055	0. 61
Benzo[*a*]pyrene	0. 045	0. 52

carbons by spectral and paper chromatographic methods. As a part of the study, however, concentrations of a number of hydrocarbons were determined. It was stressed in our previous work (*1*) that the values obtained can be regarded as minimum values only, since some of the steps, notably the picrate precipitation process, cannot be expected to be quantitative. In another study from this laboratory (*2*), the fractionation procedure was slightly modified by elimination of the picrate precipitation. The yields of hydrocarbons obtained in the second study (*2*) are in agreement with the preceding report (*1*), although the yields were slightly higher, as was to be expected. With this shortened fractionation procedure it is now possible to assay cigarette tars for 3 or 4 aromatic hydrocarbons with only 2 to 3 gm. of tar within 5 or 6 days. As in our

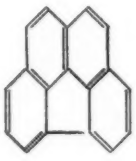
TABLE 3.—*Higher aromatic hydrocarbons in cigarette-smoke condensate**

Compound	Concentration† (*γ/100 cigarettes*)	Carcinogenicity (*8,9*)
Pyrene (*1*)	5.0 (*1*) 6.1 (*2*)	Negative
4-Methylpyrene (*1*)	5.0 (*1*)	Negative
Fluoranthene (*1*)	1.0 (*2*)	Negative
8-Methylfluoranthene (*1*)	—	Unknown
Alkylfluoranthene	—	Unknown
Chrysene	0.06	Weak
Alkylchrysene	0.04	Unknown
Benzo[*a*]pyrene (*1*) (3,4-Benzpyrene)	0.50 (*1*) 0.70 (*2*)	Strong
Methylbenzo[*a*]pyrene	0.01	Unknown
Benzo[*e*]pyrene (*1*) (1,2-Benzpyrene)	0.30	Weak
Perylene	—	Negative
Benzo[*ghi*]perylene (*1*) (1,12-Benzperylene)	—	Negative
Benzo[*mno*]fluoranthene	0.10	Unknown
Benzo[*j*]fluoranthene (7,8-Benzfluoranthene)	—	Unknown
Benzo[*k*]fluoranthene (8,9-Benzfluoranthene)	—	Unknown
1,2-Benzfluorene	—	Unknown
Dibenz[*a,h*]anthracene (1,2,5,6-Dibenzanthracene)	0.05	Strong
Benzo[*c*]phenanthrene	—	Weak

**Chemical Abstracts* nomenclature is used in this paper. The more commonly used names are given in parentheses.

†Present report and earlier studies from this laboratory.

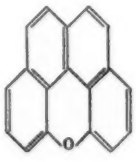
previous report, the hydrocarbons were again obtained in relatively pure form. Complete removal of materials that produced background absorption in the ultraviolet spectra was still not possible. The type of spectra obtained is exemplified in text-figure 1. In this diagram the ultraviolet absorption spectrum of benzo[*mno*]fluoranthene is given together with that of a fraction isolated from cigarette-smoke condensate. This fraction gave sharp bands corresponding to that of the authentic sample even in the 220 to 280 mμ region where background absorption sometimes obscures ultraviolet absorption spectra of column chromatographic fractions. The compound, benzo[*mno*]fluoranthene, I,



I

was synthesized by Campbell and Reid (*5*) and has since then been found in coal tar by Kruber and Grigoleit (*6*). The biological activity of this compound has not yet been determined.

1,2-Benzfluorene could not be obtained free from a compound which has the same R_F. By ultraviolet and fluorescence spectral analysis, the second component has been found to be 1,8,9-perinaphthoxanthene, II.



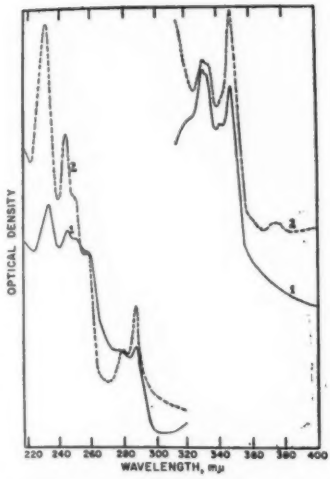
II

The spectra of this mixture are given in text-figures 2 and 3.

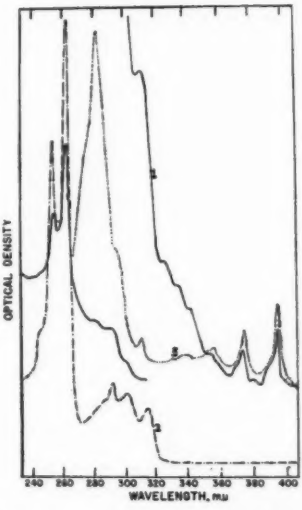
1,8,9-Perinaphthoxanthene was isolated from coal tar by Kruber (*7*) who suggested structure II for this compound on the basis of careful degradation studies, but the compound has not yet been synthesized.

It was expected that this compound would be soluble in sulfuric acid. Since the fractionation procedure used in our work involved an extraction with dilute sulfuric acid, the isolation of this heterocyclic molecule deserved further attention. It was found that the material is not soluble in 10 percent aqueous sulfuric acid at room temperature. Extraction of a benzene solution of this compound with concentrated sulfuric acid gave a yellow solution in the sulfuric-acid layer. This solution showed a cherry-red fluorescence under ultraviolet light. The fraction obtained from cigarette-smoke condensate showed the same properties.

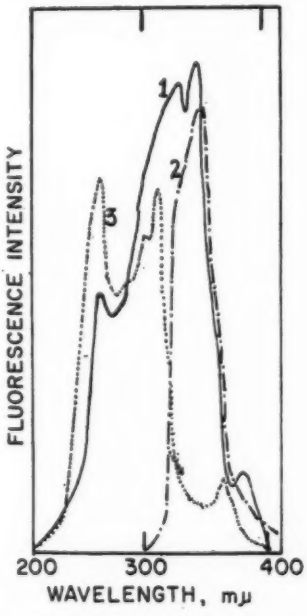
Another mixture of compounds that was obtained consisted of an alkylfluoranthene and an alkylchrysene. The fluorescence excitation and emission spectra of this mixture together with that of known model compounds are given in text-figures 4 and 5.



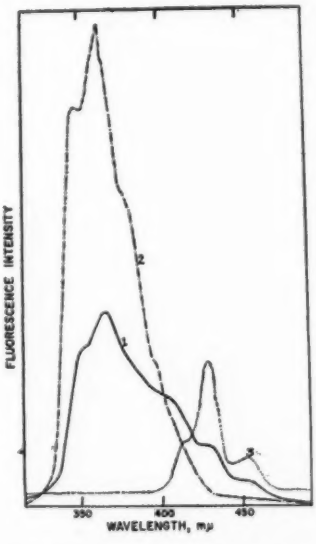
TEXT-FIGURE 1.—Ultraviolet absorption spectra: 1. Unknown. 2. Benzo[*mno*]fluoranthene. Solvent, cyclohexane.



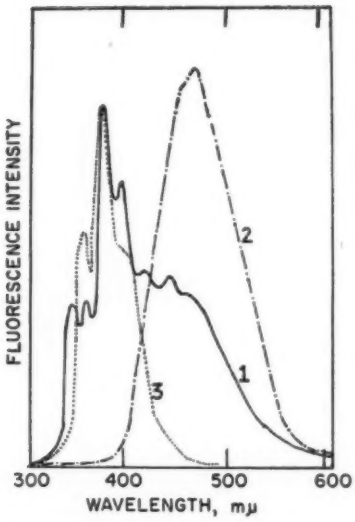
TEXT-FIGURE 2.—Ultraviolet absorption spectra: 1. Unknown. 2. 1,2-Benzfluorene. 3. 1,8,9-Perinaphthoxanthene. Solvent, cyclohexane.



TEXT-FIGURE 4.—Fluorescence excitation spectra: 1. Unknown (emission at 320 mμ). 2. 8-Methylfluoranthene (emission at 460 mμ). 3. Chrysene (emission at 380 mμ). Solvent, cyclohexane.



TEXT-FIGURE 3.—Fluorescence emission spectra, excitation at 260 mμ: 1. Unknown. 2. 1,2-Benzfluorene. 3. 1,8,9-Perinaphthoxanthene. Solvent, cyclohexane.



TEXT-FIGURE 5.—Fluorescence emission spectra: 1. Unknown (excitation at 340 mμ). 2. 8-Methylfluoranthene (excitation at 365 mμ). 3. Chrysene (excitation at 310 mμ). Solvent, cyclohexane.

Through repeated paper chromatography these 2 components were finally separated. The nature and positions of the alkyl groups on these compounds could not be determined. In both cases the relative R_F values were higher than those of the corresponding monomethyl derivatives and very similar to those of the dimethyl derivatives (table 4). Because of the large number of possible structures no attempt was made to determine the nature and positions of the alkyl groups. The alkylchrysene may be a significant component from the point of view of carcinogenic activity of the cigarette-smoke condensate. The methyl chrysenes that have been tested for carcinogenic activity are more active than chrysene (*8*). The position of the methyl group on the alkylbenzo[*a*]pyrene has also not been determined. Of the 12 isomeric methylbenzo[*a*]pyrenes, only 6 were available for comparison with the material from cigarette-smoke condensate. Of these, the 1-, 3-, 5-, 6-, and 11-methylbenzo[*a*]pyrenes were excluded on the basis of differences in R_F and/or ultraviolet absorption spectra. The unknown material had the same R_F and ultraviolet absorption spectrum as 2-methylbenzo[*a*]pyrene. The 6 other isomers are not available for comparison and cannot be excluded. 2-Methylbenzo[*a*]pyrene was tested for carcinogenicity and gave tumors in 85 days by subcutaneous injection (*8*).

With the methods developed and used in this study, it became possible to carry out a systematic search for any compound suspected of being present in our fractions. One can determine reasonably well the order of elution of a series of hydrocarbons from a column of activated alumina and one can determine exactly what the position of any given hydrocarbon

TABLE 4.—R_F *values of alkylfluoranthenes and alkylchrysenes**

Chrysene	0. 58
Fluoranthene	0. 69
6-Methylchrysene	0. 71
4-Methylchrysene	0. 71
5-Methylchrysene	0. 77
2-Methylfluoranthene	0. 79
7-Methylfluoranthene	0. 80
8-Methylfluoranthene	0. 81
5,6-Dimethylchrysene	0. 86
8,9-Dimethylfluoranthene	0. 87
Alkylfluoranthene from cigarette smoke	0. 87
1,2,3-Trimethylfluoranthene	0. 91
Alkylchrysene from cigarette smoke	0. 91
1,3-Dimethylfluoranthene	0. 91

*Relative to R_F of benzo[*a*]pyrene, 0.52.

on a paper chromatogram of a mixture of hydrocarbons would be. Using this information, a search was carried out for 1,2,3,4-dibenzophenanthrene and dibenzo[*a*,*i*]pyrene (3,4,9,10-dibenzpyrene). Both these compounds are potent carcinogens (*8–10*) and the latter has been reported in cigarette-smoke condensate (*11*). No indications could be obtained for the presence of these compounds. When any of the compounds were added to the crude fractions and chromatographed on paper, they could be recovered and identified by their ultraviolet absorption spectra. Dibenzo[*a*,*i*]pyrene, because of intense bands at 375 and 395 mμ in the ultraviolet absorption spectrum, could be detected when only 0.05 μg. was added to a crude column chromatographic fraction followed by paper chromatography. From this experiment it was concluded that this compound could be detected if it were present in a concentration of only 0.01 μg. in the tar of 100 cigarettes in our experiments.[4]

Some attempts were made to use other chromatographic procedures. Acetylated paper and alumina-impregnated paper were prepared in our laboratory by a modification of Bergmann's method (*4*). Both types of paper showed one defect, namely, uneven treatment of the paper not only from one sheet to the next but often on the same sheet. The R_F values were much lower than those obtained with untreated paper (table 2). This would suggest that the acetylated paper prepared by us is suitable for lower aromatic hydrocarbons, which have higher R_F values than the condensed 4-, 5-, and 6-ringed systems.

Evidence was obtained for the presence of a number of other hydrocarbons, some of low R_F, suggesting hexa- or heptacyclic polynuclear aromatic hydrocarbons. These compounds have not yet been identified.

[4] *Note added in proof (Sept. 10, 1958)*: Since completion of the above work, refinement of sensitivity has permitted the detection of trace amounts of dibenzo[*a*, *l*]pyrene in our cigarette-smoke condensate. In order to obtain this material we deviated from our fractionation scheme in that paper chromatography was preceded by four column chromatographies of the aromatic concentrate on activated alumina. The ultraviolet and fluorescence spectra, although sufficiently definitive for positive identification, indicated that the material obtained was not as pure as that of some of the other aromatics. Dibenzo[*a*, *l*]pyrene, according to our studies, is present in a concentration of only 0.002 μg. per 100 cigarettes. Bonnet (*11*) reports a concentration of approximately 1 μg. per 100 cigarettes.

No attempt was made to identify the lower (bi- and tricyclic) and other noncarcinogenic hydrocarbons such as anthanthrene and coronene, which may be expected to be present. The present study can therefore not be considered an exhaustive search for all the aromatic hydrocarbons in cigarette-smoke condensate. Indeed, a number of other hydrocarbons is expected to be present and some of these will be carcinogenic in nature. The compounds which are likely to be overlooked are those which do not have intense peaks in the 340 to 400 mμ region of the ultraviolet absorption spectra. It is expected that a number of alkyl derivatives in addition to those found in this study are present and that these compounds may contribute materially to the carcinogenicity of cigarette tars.

The carcinogenic hydrocarbons found so far do not by themselves appear to be present in sufficient concentrations to account for the observed activity. In tobacco tar there are many possible promoting agents. For example, the esters of long-chain fatty acids with various polyalcohols have been considered to be cocarcinogens (*12*). In another report we described the identification of a number of long-chain fatty acids in cigarette smoke (*13*) and found that these acids are present partly as esters. Other types of carcinogenic compounds, as yet unknown, may, of course, also be present.

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Mammary-Cancer-Inducing and Inhibitory Inherited Hormonal Patterns in Mice [1,2]

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Summary

By mating females of the cancerous A stock, which have a low incidence of mammary cancer as virgins, with males of the CBA, C (BALB/c), and I^a strains, it was then determined that these 3 strains transmitted the mammary-cancer-inducing inherited hormonal pattern. This hormonal mechanism will produce a high incidence of mammary cancer in susceptible females that are maintained as virgins when they possess the mammary-tumor agent (MTA). Mice of the JK and I^a strains were found to be resistant to the development of mammary cancer. This conclusion was reached after observing females, which acquired the MTA by nursing females of cancerous stocks, and breeding them. Members of the JK stock, although they develop postcastrational adrenocortical hyperplasia, possess an inherited hormonal pattern that presumably either inhibits or delays the development of mammary tumors even in susceptible females with the MTA kept as breeders. Observations of certain crosses could not be explained on any physiological or genetic basis, unless there may be synergistic action between certain of the hormonal patterns acting to influence the genesis of mammary cancer in both breeders and nonbreeders.—**J. Nat. Cancer Inst. 21: 631–640, 1958.**

Until recently it was generally presumed that ovariectomy would interfere with the development of mammary cancer in mice and that the younger the females were at the time of the operation, the lower the incidence would be (*1–4*). However, in 1939, Woolley, Fekete, and Little (*5–8*) reported that the removal of ovaries from day-old mice of one cancerous strain did not significantly reduce the incidence of mammary tumors, though the average cancer age was delayed. For controls, they observed females that had one ovary extirpated. The disease also was observed in a few castrated males.

[1] Received for publication April 24, 1958.

[2] This work was supported by research grant C-372 from the National Cancer Institute, National Institutes of Health, Public Health Service; a grant-in-aid from the American Cancer Society, Inc., upon recommendation of the Committee on Growth, of the National Research Council; the Minnesota Division of the American Cancer Society; the Elsa U. Pardee Foundation; and the Graduate School Cancer Research Fund of the University of Minnesota.

In 1944 (*9, 10*) it was determined that the hormonal pattern or mechanism required for the genesis of mammary cancer in virgin or nonbreeding mice was transmitted. In these studies strain A mice that were susceptible to mammary cancer and had a low incidence of the disease in virgins (*11*) were crossed with mice of the cancerous C3H strain in which the nonbreeders had a high incidence (*10*). In the reciprocal F_1 females that possessed the mammary-tumor agent and were maintained as virgins, high incidences of mammary tumors were observed, demonstrating that the mammary-cancer-inducing hormonal pattern, originally termed the "inherited hormonal influence" (*12*), was transmitted by both males and females.

Nodular hyperplasia of the adrenal cortex had been observed by Woolley *et al.* in ovariectomized females of the dilute brown (DBA) (*5, 8*) and C3H (*6*) strains, and adrenal carcinoma in gonadectomized animals of the CE stock (*13–19*), while the ovariectomized females of C57 black (C57BL) (*6*) and cancerous A (*20*) strains remained in the castrate state. More recently (*20, 21*) the data were extended to include basophilic adenomas of the pituitary in castrated mice of certain strains and their hybrids, in addition to the adrenal lesions.

These and other (*22*) experimental observations suggested (*12*) that analogous factors might be responsible for the postcastrational adrenal alterations and the inherited hormonal pattern associated with the induction of mammary cancer in virgin females (*9, 10*). Such a relationship was established in this laboratory by testing virgin and ovariectomized females of the A and Z (C3H) strains and their reciprocal hybrids, with and without the mammary-tumor agent (MTA) (*12, 23–25*). Since that time many publications have been concerned with either the physiological effects of the hormonal mechanisms or their genetic aspects (*25–39*). Additional information for other inbred strains and their hybrids are presented in this report.

Materials and Methods

Strong (*40–43*) has reported on the origin of all strains employed in these experiments except the C (BALB/c) stock (*44, 45*), and details concerning the development of the author's agent-free sublines of strains A and Z (C3H), which are susceptible to cancer, have been given previously (*11, 12, 25–37*). Mice of 2 sublines of the I stock were tested, one, the I^b line, has been used in many studies on mammary cancer (*4, 29–31, 46–52*), while the other, the I^a line (*4*), was obtained from Strong in 1950. Representatives of the C stock were secured from Snell in 1942 (*45*), JK mice from Strong (*53*) in 1946, and CBA animals from Kirschbaum (*54*) in 1951.

Because males of the I^b subline transmitted a hormonal mechanism that induced mammary tumors in virgin AI^bF_1 females (*29–31, 52*), males of the I^a line were tested and crosses were made between females of the A strain and males of the CBA and C stocks after Frantz and Kirschbaum (*54*) observed adrenocortical hyperplasia in ovariectomized females of these strains.

Experimental Results and Discussion

The observations given in table 1 for F_1 females maintained as nonbreeders show that the 3 inbred strains used in the present studies, or the CBA, C, and I^a stocks, all transmitted the mammary-cancer-inducing hormonal pattern. Data for the AI^bF_1 hybrids are presented for comparison; figures previously reported (*31*) together with unpublished data were used since the results were similar.

It has not been established whether CBA mice might be susceptible to the development of mammary cancer. Tumors have not been found in unfostered breeders of the strain, but mammary tumors are appearing in CBA females that had been nursed by females of the cancerous Z stock to obtain the MTA. Of 52 breeders of the I^a line, only 1 had a mammary tumor when they had the agent, an incidence comparable to that seen in members of the I^b line (*4, 29, 31, 48–50*). High incidences of mammary tumors have been found in agent-bearing C females, which demonstrates that they are susceptible to spontaneous mammary cancer (*55–57*).

That the I^a line transmitted the inducing hormonal pattern was of particular interest since the 2 sublines of the I stock have been separated and maintained in different laboratories for more than 20 years. Mice of both lines are resistant to the development of spontaneous mammary cancer. They do not effectively propagate and transfer the MTA. The chinchilla-coat-color genes are carried by the I^b but not I^a mice, and yet animals of both lines possess the inducing hormonal pattern for mammary cancer. That there also may be an accelerating factor like that recorded for the I^b hybrids (*31*) may be indicated from the early average cancer age seen in the AI^a F_1 females—nearly 100 days (234 *vs.* 330 days) earlier than that of breeders in the cancerous A stock. The average age for AI^bF_1 breeders was 224 days (*31*).

TABLE 1.—*Development of mammary cancer in F_1 females obtained by mating females of the A strain with males of the C(BALB/c), CBA, and I^a stocks**

Stock or generation	Number of mice	Observations on virgin females		
		Cancer (*percent*)	Average age (*days*)	
			Cancerous	Non-cancerous
A strain	111	4	450	573
ACF_1	51	75	474	671
$ACBAF_1$	45	78	530	535
AI^aF_1	82	65	438	707
AI^bF_1*	77	73	463	713

*Data for AI^bF_1 hybrids represent published (*31*) and unreported observations.

Animals of the inbred I^a line and hybrid generations were given to Martinez to determine what their postcastrational adrenal response might be, but these data are not available at this time.

F_1 hybrids produced by mating males of the C stock with females of the A strain had a high incidence of mammary tumors when continued as nonbreeders (table 1). As in other similar cases, postcastrational adrenal alterations were observed in mice of the paternal strain (*54*).

The basis for testing mice of the CBA stock was their reaction after gonadectomy since Frantz and Kirschbaum *(54)* found postcastrational adrenal hyperplasia with hormonal stimulation of the secondary sex organs. As shown by the cancer incidence of 78 percent (table 1) in virgin F_1 (A♀ × C♂) females, the association was again established between the inducing inherited hormonal mechanism and the effects of castration, which has been considered previously (*4*, *36*, *37*).

Mühlbock (*58*) reported that F_1 females obtained by mating A♀ × CBA♂ would give rise to mammary tumors even when their progeny were kept as virgins. No mention was made, either by the author or by those who discussed the data when it was presented, of the possibility that the action of any inherited inducing hormonal pattern contributed by the CBA animals might be responsible for the high incidence in the F_1 virgins.

After ovariectomy, females of the JK stock (*53*) will show adrenocortical lesions, and estrus will appear in young mice due to the production of an abnormally large amount of estrogenic hormone (Huseby and Bittner, unpublished). As in other studies, males of the JK strain were mated with females of the A stock to ascertain the influence of their hormonal mechanism upon the development of mammary tumors in the hybrids. Preliminary data have been mentioned in earlier reports (*28*, *34*), with the final results tabulated in table 2.

Six JK females were nursed by females of the cancerous Z stock so that they would obtain the MTA, and 14 females of the following 2 inbred generations were observed, all as breeders. Two of these JK females had mammary cancer; 1, a member of the fostered generation, at an average cancer age of 282 days. The noncancerous females survived to an average age of 511 days.

In the F_1 hybrids obtained from crossing A females with JK males, and the 2 backcross generations to the parental stocks (AxBC and JKBC), not more than 17 percent of the virgins in any group had mammary cancer. Only 50 percent of the $AJKF_1$ breeders died of cancer; this incidence did not increase significantly in the backcross to the maternal A stock and decreased to 22 percent in breeders of the backcross generation to the JK stock (table 2). In only one other cross (*32*) was the incidence lower in F_1 hybrids than in the maternal A stock when breeders were observed (*4*, *34*).

$AJKF_1$ females also were mated with males of the Zb, I^b, and CE stocks, and the resulting hybrids may be designated only by matings. Members of every group had higher incidences of mammary tumors than

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TABLE 2.—*Observations on development of mammary cancer in hybrids produced by crossing animals of inbred A and JK stocks, and others derived by mating AJKF$_1$ females with males of Zb, I^b, and CE stocks*

Stock or generation	Matings ♀ × ♂	Observations on breeding females				Observations on virgin females			
		Number of mice	Cancer (*percent*)	Average age (*days*)		Number of mice	Cancer (*percent*)	Average age (*days*)	
				Cancerous	Noncancerous			Cancerous	Noncancerous
A	A × A	569	84	330	387	111	4	450	573
AJKF$_1$	A × JK	98	50	479	673	115	17	651	725
AJKF$_1$-AxBC	AJKF$_1$ × Ax	55	64	359	574	87	17	491	673
AJKF$_1$-JKBC	AJKF$_1$ × JK	22	22	404	651	19	11	625	646
	AJKF$_1$ × Zb	47	66	315	600	75	75	444	726
	AJKF$_1$ × I^b	40	75	326	515	30	43	518	719
	AJKF$_1$ × CE	41	76	366	580	41	44	513	736

did mice of the Ax or JK backcross generations, with an acceleration in the time of appearance of tumors in 2 of the groups. When Zb males were mated with $AJKF_1$ females, their progeny, kept as virgins, showed an incidence as high as that of mice of any other group observed as breeders (table 2).

When they possess the MTA, nearly 90 percent of the breeders of the CE stock will develop mammary cancer, while only 26 percent of the nonbreeders had tumors (*4, 33, 34, 59*). On the other hand, mice of the 2 sublines of the I stock had low incidences even as breeders and did not propagate the agent for more than a few generations (*4, 31, 34, 49–51*). If CEz (CE females with the Z agent) were mated with I males, regardless of subline, 86 percent of the F_1 virgin progeny and 96 percent of the F_1 breeders had mammary tumors at the respective average ages of 347 and 302 days (*4*).

In the cross between females of the A strain and CE males, only 37 percent of the $ACEF_1$ virgin females had tumors at an average age of 568 days (*33*), compared with incidences above 65 percent with early cancer ages for the virgin hybrids that resulted from the A♀ × I♂ crosses (table 1). A difference in excess of 100 days was found in the average ages of the cancerous F_1 breeders.

With the apparent differences between mice of the CE (*33, 59*) and I^b (*4, 29, 31, 48–50*) stocks, involving both the inherited susceptibilities to mammary tumors and the inherited hormonal patterns, it would be difficult to account for the observations obtained by mating $AJKF_1$ females with either I^b or CE males (table 2). The progeny of these crosses had similar incidences and average cancer ages, whether they were continued as breeders or as virgins, and the breeding groups had higher incidences than the offspring born to $AJKF_1$ females with Zb fathers or sired by males possessing both the inherited susceptibility and the inducing hormonal mechanism (*9, 10, 12, 25*). When the hybrids had I^b and CE fathers and were continued as nonbreeders, the incidences were nearly as high as in breeders of the $AJKF_1$ generation (table 2).

Comparisons may be made between the offspring produced by mating I^b males with $AJKF_1$ females (50% in breeders and 17% in virgins, table 2) and AI^bF_1 females [95% in breeders and 72% in virgins (*31*)]. The respective average cancer ages for the $AJKF_1$ and AI^bF_1 hybrids were: breeders, 479 and 224 days; nonbreeders, 651 and 451 days. In spite of the differences between the F_1 parents, the respective incidences in the breeding and virgin progeny of the cross between $AJKF_1$♀ and I^b♂ were 75 percent and 43 percent; the respective incidences for the AI^bF_1–I^bBC hybrids were 56 percent and 44 percent (*31*). No explanation for these data may be offered other than the possibility of some synergistic effect of the hormonal patterns transmitted by the parents. Even this would not account for the excessive high incidences in the hybrid breeders with I^b and CE fathers (table 2), since the I^b mice have been shown to be resistant to the development of mammary cancer (*4, 31, 34, 48–51*) and CE mice are susceptible (*33, 34*).

In an attempt to account for these observations, 2 other crosses involving CE mice were made. Twenty-two additional CE females were fostered by females of the cancerous Z strain of which 86 percent developed cancer at an average age of 420 days; the incidence in their progeny and descendants was nearly as high. These CEz females were mated with JK, I^b, and B_1 (C57BL)F_1 males [*see* (*4*) and (*34*)]. The incidences of mammary tumors in breeding and virgin $CEzB_1F_1$ females are 60 percent [8] and 11 percent, with 24 percent and 67 percent of the respective groups still living after the age of 17 months. Hybrids with CEz mothers but sired by JK fathers have had an incidence of 59 percent in breeders and 36 percent in nonbreeders. Thus, the $CEzJKF_1$ mice do not have a higher incidence of mammary tumors than breeding $CEzB_1F_1$ hybrids, and both incidences were lower than recorded in the hybrids sired by the resistant I males but with CEz mothers, considered previously.

As discussed in earlier publications (*31*), the same factors are not involved in the transmission of the inherited susceptibility to development of mammary cancer and the inherited hormonal pattern that induces the disease in virgin females. A survey of all the experimental data would show that there are several other inherited hormonal mechanisms, one apparently inhibiting the development of mammary cancer in mice, which have other causative factors. An inhibitory hormonal pattern may be transmitted by animals of the JK stock (table 2), perhaps similar to that found for the NH strain (*32*, *34*, *35*). Whereas the NH mice are probably susceptible to mammary tumors, the observations obtained in the present studies would indicate that the JK mice are relatively resistant. Further experimentation must be made in an attempt to ascertain why the progeny of certain hybrid crosses have excessive incidences of mammary tumors when the offspring descended from the parental stocks have low incidences in other crosses.

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Studies on the Anemia of Tumor-Bearing Animals. I. Distribution of Radioiron Following the Injection of Labeled Erythrocytes [1]

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Summary

Iron distribution studies on rats bearing certain transplanted tumors indicated that the tumors contained large amounts of iron, frequently exceeding the total amount of iron found in the liver. Following the intravenous injection of Fe^{59} labeled red cells into normal and tumor-bearing rats and mice, the Fe^{59} concentration decreased at a more rapid rate in the tumor-bearing animals. The rate at which the Fe^{59} concentration decreased, and the time after implantation of the tumor when the decrease started, varied markedly from tumor to tumor. The rate at which the Fe^{59} left the blood of any single tumor-bearing animal varied randomly from day to day. The tumor-bearing animals frequently had larger blood volumes than those of their normal controls. In some instances the increased blood volume of the tumor-bearing animal was associated with an increased total body weight. In no instance did the change in blood volume more than partially account for the decrease in the concentration of labeled erythrocytes observed in the circulating blood of the tumor-bearing animals. A study of the distribution of Fe^{59} in the normal and in the tumor-bearing animals revealed that a high proportion of the Fe^{59} which had left the blood stream could be found in the area of the tumor. The loss of Fe^{59} labeled erythrocytes from the blood stream was correlated with the amount of Fe^{59} in the tumor, the onset of anemia, and the development of cachexia in the host.—**J. Nat. Cancer Inst. 21: 641–656, 1958.**

Most patients with cancer have an anemia at some stage of their disease. In some instances the anemia obviously results from hemorrhage externally or into the gastrointestinal tract. In most patients, however, the anemia cannot be attributed to this cause (*1*). The etiology of anemia in the patients who have no overt hemorrhage is a problem that has been of concern to physicians for many years. Early investigators thought it resulted from a crowding out of the bone marrow by neoplastic tissue. In recent years, however, it has become increasingly apparent that in leukemic patients a process of erythrocyte destruction

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has a dominant role in the pathogenesis of the anemia (*2–5*). In the past decade many clinical investigators have found a shortened red cell survival time in patients with leukemia (*6–15*) and with solid tumors (*6, 16–20*), frequently in the presence of normal or even increased rates of erythropoiesis (*19, 21–23*). These studies have established the importance of the destructive process in the pathogenesis of the anemia.

Although the importance of red cell destruction is becoming more and more apparent, in most instances the nature of the destructive process has remained obscure despite numerous investigations. These investigations have been directed primarily toward 1) studies on tumor extracts for evidence of hemolytic substances which might cause injury or lysis of red cells upon release into the circulation (*24–31*), and 2) studies on the erythrocytes of patients and animals with cancer for evidence of injury (*1, 7, 15, 32–36*) or of intrinsic defects (*11, 37*). Although substances that are hemolytic *in vitro* can be isolated from tumors, evidence is not yet available that they are released into the circulation and are important in the destruction of red cells in the cancer patient (*31, 38, 39*). Evidences of extrinsic defects of erythrocytes, such as the presence of spherocytosis, a positive Coombs test, or of an increased hypotonic fragility, have been demonstrated in some animal tumors (*35*), and are fairly common in patients with chronic lymphatic leukemia (*36*) and with ovarian tumors (*32–34*). However, Shen and Homburger found evidence of a positive Coombs test or increased osmotic or mechanical fragility in only 3 out of 193 patients with carcinoma. Satisfactory evidence of intrinsic defects due to malformation of red cells in cancer patients is still lacking (*39*). Eadie and Brown, in a review of red cell survival studies, have pointed out, however, that random destruction of red cells may occur as the result of either hemolysis or hemorrhage (*40*).

In the course of tracer studies with Fe^{59} on the turnover of liver catalase in tumor-bearing animals, the authors found a large amount of iron in the tumor which was not accounted for by the iron of the circulating blood (*41*). It was also noted that the amount of iron in the tumor was nearly balanced by a decreased amount of iron in the circulating blood. These observations raised the question whether the erythrocyte destruction occurring in the cancer-host might not be secondary to vascular injury rather than from direct injury to the red cells by a tumor toxin. Since quantitative studies on this phenomenon would be difficult in the human patient, it seemed that the anemic tumor-bearing rats and mice might provide an experimental system in which to test this hypothesis. The results of these studies [3] are presented herein and in the two following reports (*42, 43*) of this series.

Materials and Methods

Experimental techniques.—Labeled red cells were obtained by the intraperitoneal injection of approximately 100 microcuries (μc.) of Fe^{59}

[3] The results of part of this investigation were presented at the 47th annual meeting of the American Association for Cancer Research, Atlantic City, N. J., April, 1956.

as ferric ammonium citrate, per 100 gm. of body weight, into 1- to 2-month-old rats.[4] At the end of 24 to 48 hours, blood was withdrawn from the bifurcation of the aorta into a heparinized syringe. The cells were washed 3 times with normal saline; the final dilution was such that the desired counts to be injected were contained in a volume of 0.25 or 0.50 ml.

The Cr^{51} tagged cells were prepared by the incubation of heparinized blood with radio-chromate [5] (*44*). The amount of Cr added ranged from 5 to 20 μg. per ml. of blood. The blood was incubated for 2 hours with radio-chromate and then washed twice with normal saline.

Animals to be injected with labeled cells were anesthetized with 4 mg. of sodium pentobarbital per 100 gm. of body weight. The femoral artery and vein were exposed and two ligatures were placed under them, one proximal and one distal to the site of injection. The needle was inserted into the vein, and the infusion of labeled cells was observed through the wall of the vein. On completion of the injection, both the proximal and distal ligatures were tied, the needle was withdrawn, and the incision closed with skin clips. Although this procedure did not interfere with the function or growth of the leg in the rat, some atrophy of the leg was observed in mice.

Small blood samples for the periodic measurement of hemoglobin and radioactivity were obtained by snipping a small section from the tail of the rat. The blood was drawn into a calibrated pipette of about 15 μl. volume, which had previously been rinsed with a 1 percent solution of heparin. The blood sample was pipetted into 2 cc. of 0.01 percent Na_2CO_3 (*45*) in a 4 cc. counting vial; the tip of the tail was cauterized with a soldering iron. This procedure could be repeated many times with a loss of less than 50 μl. of blood at any given bleeding. The optical density of the hemoglobin solutions was read at 580 mμ. At this wavelength, by using standard Beckman cuvettes with a 1 cm. light path, an optical density of 1.0 is equivalent to 1 mg. of hemoglobin per ml.

All Fe^{59} and Cr^{51} counting was made in a well-type scintillation counter.[6] The samples were counted in 2 ml. of fluid in a 4 ml. counting vial as described by Weisburger *et al.* (*46*). With this counting technique, 1 μc. of Fe^{59} gave approximately 1×10^6 counts per minute (c.p.m.), or 45 percent efficiency, and Cr^{51} gave 1.18×10^5 c.p.m., or about 5 percent efficiency. The data presented have routinely been corrected for decay back to the day of injection of the isotope into the animal.

The energy difference between the *gamma* irradiation of Fe^{59} and Cr^{51} permitted simultaneous counting of these two isotopes in the same sample. Curves were made for Fe^{59} and Cr^{51} relating counts to voltage. From these curves a point was selected at 1200 volts which lay on the counting plateau of both Fe^{59} and Cr^{51}. A second point at 900 volts was selected. At this point less than 1 percent of the chromium counts obtained at 1200 volts

[4] In some of the later experiments 100 μc. was divided into 20 μc. doses administered twice daily, and the animals were sacrificed on the 3rd to 5th day. The degree of incorporation and the experimental results obtained after the injection of cells prepared in this way were essentially the same as those obtained with cells labeled as described.

[5] Sodium radio-chromate solution, Rachromate ®, Abbott Laboratories, North Chicago, Ill.

[6] Model DS-3 Nuclear Instrument and Chemical Corporation, Chicago, Ill.

was recorded but approximately 50 percent of the iron counts was still registered. By routinely counting an Fe^{59} standard each time the voltage was set at either 900 or 1200 volts, the exact ratio of counts at the two voltages was determined. From this ratio, the number of counts at 1200 volts due to iron was calculated from the count obtained at 900 volts. The chromium count at 1200 volts was determined by subtracting the calculated iron counts from the total counts at 1200 volts.

Tumors.—Lymphosarcoma R2788 (provided by Dr. Wilhelmina F. Dunning, *47*) was carried subcutaneously in F_1 hybrids of Marshall 520 females crossed with A × C/9935 males. The tumor was relatively free of gross hemorrhage and attained a fairly large size before necrosis was apparent. The tumors grew in 100 percent of the injected animals, and death usually occurred in 3 to 4 weeks.

Hepatoma 3683 (given to us by Dr. Harold P. Morris, *48*) was transplanted intraperitoneally into F_1 hybrids of M520 × (A × C/9935) rats. A nodular tumor with a moderate amount of hemorrhagic ascitic fluid was obtained, which killed the animal in 3 to 4 weeks.

The Yoshida ascites tumor (provided by Dr. Morris Belkin and Dr. Edward S. Essner, *49*) was transplanted by the intraperitoneal injection of 0.05 cc. of ascitic fluid into M520 rats. The ascitic fluid contained increasing numbers of erythrocytes after the 7th day and usually killed the animal within 10 days to 2 weeks.

The following tumors were specifically selected because of their lack of hemorrhage until late stages of growth: the Harderian gland carcinoma 2226 (*50*) and Fibrosarcoma 4956 (both provided by Harold P. Morris) carried subcutaneously in Buffalo rats; Fibrosarcoma HE8971 (furnished by Dr. Walter E. Heston, *51*), carried subcutaneously in C3H/He mice. The H134 ascites hepatoma (obtained from Dr. Belkin and Dr. Essner, *52*) was transplanted intraperitoneally by the injection of 0.05 ml. of ascitic fluid into C3H/He mice.

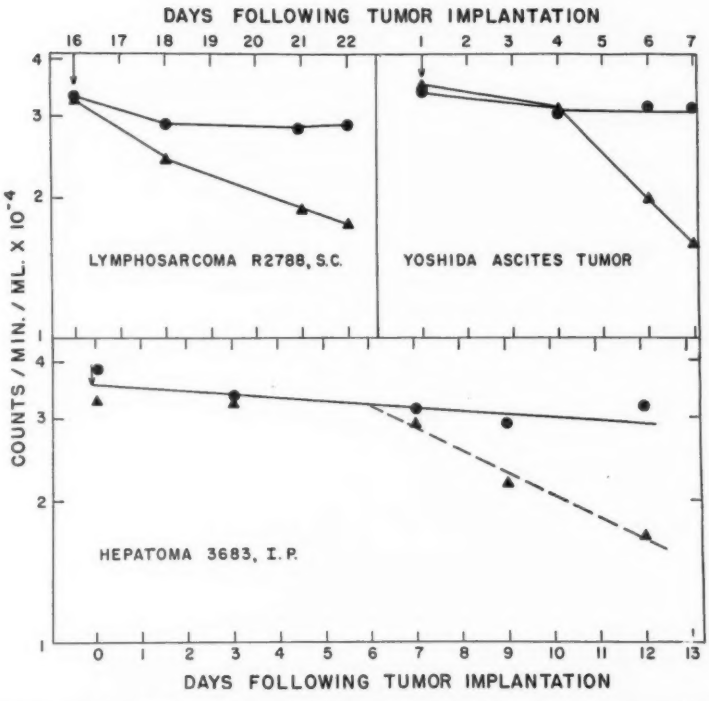
Spontaneous mammary tumors in C3H mice were provided by Dr. H. B. Andervont.

All the experimental animals were fed *ad libitum* with Bryant mouse pellets prepared from a formula containing 1.3 mg. of iron citrate per gm. of pellet (*53*).

Experiments

Survival of Fe^{59} Labeled Erythrocytes in Tumor-Bearing Animals

In order to measure the random destruction of red cells in rats bearing Lymphosarcoma R2788, 0.25 ml. of Fe^{59} labeled red cells containing 0.33 μc. of activity were injected intravenously into both normal and tumor-bearing rats. Blood samples, on which hemoglobin and Fe^{59} determinations were made, were taken from the tail at intervals. The results are plotted in text-figure 1. The concentration of Fe^{59} in the peripheral blood of normal animals showed a decrease of 15 percent of the labeled red cells


DAYS FOLLOWING TUMOR IMPLANTATION
16 17 18 19 20 21 22
1 2 3 4 5 6 7
COUNTS / MIN. / ML. X 10⁻⁴
LYMPHOSARCOMA R2788, S.C.
YOSHIDA ASCITES TUMOR
HEPATOMA 3683, I.P.
0 1 2 3 4 5 6 7 8 9 10 11 12 13
DAYS FOLLOWING TUMOR IMPLANTATION

TEXT-FIGURE 1.—Fe^{59} counts in the peripheral blood following the injection of labeled erythrocytes. ↓ = point of injection of labeled cells. ● = normal control animals. ▲ = tumor-bearing animals. The Yoshida ascites tumor averaged 37 ml. of ascitic fluid on the 7th day following intraperitoneal injection.

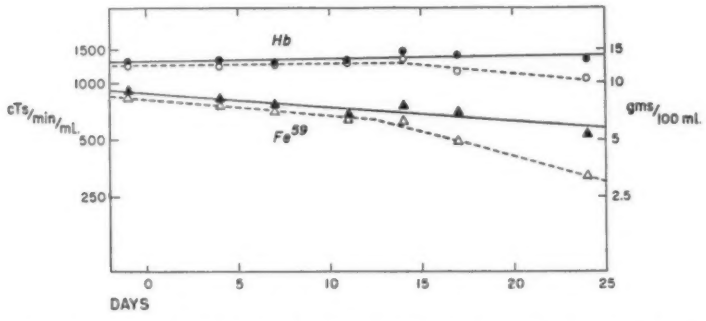
in the 6 days following their injection, whereas the tumor-bearing animals showed a decrease of 46 percent of the labeled cells.[7]

When Hepatoma 3683 was implanted intraperitoneally only 1 day before labeled cells containing 0.34 μc. of Fe^{59} were injected (text-fig. 1), little change in the concentration of the labeled red cells in the blood was observed during the early stages of tumor growth. As the tumor grew larger, however, a pronounced progressive fall in the concentration of labeled cells occurred.

The Yoshida ascites tumor (text-fig. 1) was also injected one day before labeled cells containing 0.30 μc. of activity were injected. The marked decrease in the concentration of radioactivity began at about the 4th day. At the end of 7 days the concentration of labeled cells had decreased by 55 percent in the tumor-bearing animals, while the concentration in the normal animal had decreased only 8 percent.

[7] It should be pointed out that the curves presented in this section reflect any changes in blood volume as well as any erythrocyte destruction that is occurring. It is not possible to correct for changes in blood volume in these curves, but the total change in blood volume during the experiment has been determined and is presented in a later section.

When Fe^{59} labeled red cells containing 0.42 μc. of activity were injected into rats bearing the Harderian gland carcinoma 2226, the decrease in the concentration of labeled cells (text-fig. 2) occurred later and at a slower rate than that observed with the previous tumors. The concentration of Fe^{59} in the tumor-bearing animal continued to fall at the same rate as in the normal controls during the first 2 weeks, though the tumor was growing rapidly and was already large at the end of this period. The fall in radioiron concentration in the normal controls reflects growth of the animals, with a corresponding increase in blood volume.



TEXT-FIGURE 2.—Fe^{59} counts in the peripheral blood of normal rats and rats bearing the Harderian gland carcinoma 2226 following the injection of Fe^{59} labeled erthrocytes. △ and ○ = Fe^{59} and hemoglobin concentrations, respectively, of the normal controls. ▲ and ● = Fe^{59} and hemoglobin, respectively, of the tumor-bearing animals.

In order to determine whether a similar loss of labeled cells occurred in spontaneous tumors, 0.1 ml. of labeled cells containing .047 μc. of Fe^{59} was injected into C3H mice bearing small, spontaneous mammary carcinomas. The loss of labeled cells and the development of anemia began early but continued at a slow rate until the 17th day of tumor growth, after which time the rate of loss was accelerated.

Changes of Body Weight in Tumor-Bearing Animals

Animals bearing Lymphosarcoma R2788 and Hepatoma 3683, and in which there was a rapid decrease in the concentration of labeled cells in the peripheral blood, were also characterized by a loss of carcass weight and an early onset of cachexia. The final total body weight of these animals (table 1) was not appreciably different from the control animals. However, the carcass weight, obtained by subtracting the tumor weight from the final body weight, was reduced 17 to 24 percent in the animals bearing these tumors. The emaciation of the tissues surrounding the skull and vertebral column, the marked weakness, and the mincing gate attested to the severity of the cachexia in these animals.

In marked contrast, rats bearing Carcinoma 2226 and mice bearing the spontaneous mammary carcinoma, and in which there was a more gradual

decrease in the labeled cells of the peripheral blood, were alert and active, and showed little evidence of cachexia until very late. In these animals the total body weights were much larger than the body weights of the normal controls (table 1). Although the tumors were large, the carcass weights were not significantly different from the body weights of the controls. If allowed to grow, Carcinoma 2226 often reached 50 percent of the total body weight before death occurred.

TABLE 1.—*Effect of tumors on body weight*

Host	M520 × (A × C) rats				Buffalo rats		C3H mice	
Tumor	Lymphosarcoma R2788		Hepatoma 3683		Carcinoma 2226		Spontaneous mammary carcinoma	
	N*(2)†	T(4)	N(2)	T(3)	N(2)	T(3)	N(3)	T(2)
Initial body weight‡	235	225	200	217	154	151	29. 5	35. 0
Final body weight	243	232	195	199	203	284	29. 5	37. 0
FBW-IBW	+8	+7	−5	−18	+49	+133	0	+2. 0
Tumor weight	—	30	—	29	—	68	—	3. 8
Carcass weight	243	202	195	170	203	216	29. 5	33. 2
CW-IBW	+8	−23	−5	−47	+49	+65	0	−1. 8
Rate of RBC destruction		Rapid		Rapid		Slow		Moderate

*N = normal control rats. T = tumor-bearing rats.

†Figures in parentheses designate the number of animals in each group.

‡Weights obtained at the beginning of each experiment. Although usually coinciding with the transplantation of the tumor, there were certain exceptions: the lymphosarcoma was transplanted 16 days before the injection of labeled cells, and the spontaneous mammary tumor was injected with labeled cells after the tumor was already of moderate size.

Changes of Blood Volume in Tumor-Bearing Animals

In order to determine to what extent a change in blood volume accounted for the decreased concentration of labeled cells in the peripheral blood during these experiments, an initial and a final blood volume determination was made. The initial blood volume was obtained by measuring the dilution of Fe^{59} labeled cells in the red cell space after a 30-minute period of equilibration. The final blood volume was similarly obtained by measuring the dilution of Cr^{51} labeled cells injected 30 minutes before the animal was killed. These data are presented in table 2.

The animals bearing Lymphosarcoma R2788 had a considerable increase in blood volume amounting to about 30 percent, and those with Hepatoma 3683 had a moderate increase of about 20 percent. Those bearing the Harderian gland carcinoma 2226 had a considerable increase in total blood volume, which was proportional to the increase in body weight, so that the blood volume expressed in terms of ml. per 100 gm. of body weight was not significantly altered. The mice bearing spontaneous mammary tumors had a remarkable increase in blood volume with only

a small increase in body weight. In these animals a tumor weighing 10 percent of the body weight caused an increase in blood volume of nearly 50 percent. The numerous dilated sinusoids seen in this tumor on microscopic examination indicate that the elevated blood volume may result in large part from the marked vascularity of these tumors.

TABLE 2.—*Effect of tumors on blood volume**

Tumor type	Lympho-sarcoma R2788		Hepatoma 3683		Carcinoma 2226		Spontaneous mammary carcinoma	
	N	T	N	T	N	T	N	T
Total blood volume (*ml.*)								
Initial	10. 5	9. 6	8. 9	10. 4	7. 3	7. 7	1. 6	1. 9
Final	10. 5	12. 9	9. 0	11. 8	11. 0	15. 2	1. 4	2. 9
Change	0. 0	+3. 3	+0. 1	+1. 4	+3. 7	+7. 5	−0. 2	+1. 0
Ml. per 100 gm. body weight								
Initial	4. 5	4. 3	4. 5	4. 8	4. 7	5. 1	5. 4	5. 4
Final	4. 3	5. 6	4. 6	5. 9	5. 4	5. 4	4. 8	7. 8
Change	−0. 2	+1. 3	+0. 1	+1. 1	+0. 7	+0. 3	−0. 6	+2. 4
Percent change	−4	+31	+2	+23	+15	+6	−11	+44

*Additional information on these animals is presented in table 1.

It will be seen that during the period of study all the tumor-bearing animals had an increase in blood volume due to either growth of the tumor or the host or to an increase in specific blood volume of the tumor as compared to that of the host. Any increase in blood volume will cause a corresponding dilution of labeled cells in the circulating blood. In text-figures 1 and 2, some of the fall in the concentration of labeled cells results from this increase in blood volume and not from destruction of red cells. Therefore, in studies on tumor-bearing animals, it is important to carry out a second blood-volume determination at the conclusion of each study to evaluate the change in blood volume, and to estimate the amount of radioisotope injected that is still in the circulating blood and the amount that has actually left the blood stream. Data on the recovery of the radioisotope from the various organs will be presented in the next section.

Distribution of Fe^{59} in Various Organs

The distribution of Fe^{59} in various organs of the normal and tumor-bearing animals was determined after the preceding experiments were completed. The Fe^{59} content of the circulating blood remaining in each organ after decapitation and exsanguination of the animal and removal of the organ was calculated by the following formula: Cr^{51} content of the organ $\times$ ratio of Fe^{59}/Cr^{51} in the circulating blood = amount of circulating Fe^{59} remaining in the organ. The ratio of the concentration of Fe^{59} to Cr^{51}

in the blood was obtained on a tail sample just before the animal was killed. The amount of noncirculating radioiron was obtained by subtracting the circulating radioiron from the total radioiron content of the tissue. The distribution of noncirculating Fe^{59} in the tissues of animals bearing Lymphosarcoma R2788, Hepatoma 3683, and Yoshida ascites tumor is presented in table 3.

TABLE 3.—*Distribution of Fe^{59} counts in tumor-bearing rats following injection of labeled red cells**

Host	M520 × (A × C)		M520 × (A × C)		M520	
Tumor	Lymphosarcoma† R2788, S.C.		Hepatoma† 3683, I.P.		Yoshida ascites‡	
	N	T	N	T	N	T
Blood	90. 2	67. 0	84. 6	58. 4	89. 7	50. 3
Tumor	—	17. 3	—	21. 4	—	23. 8
Liver	1. 6	4. 5	1. 2	5. 4	4. 8	14. 3
Spleen	—	—	6. 3	4. 4	—	—
Recovery	91. 8	88. 8	92. 1	89. 6	94. 5	88. 4

*Expressed in terms of percent of injected counts.
†*See* table 1 for tumor weights.
‡*See* text-figure 1 for tumor weight.

The total radioiron recovered in the blood indicated that 33 to 50 percent of the labeled cells had left the circulating blood of the tumor-bearing animals, while only 10 to 15 percent had left the blood of the normal animals over the same period of time. Sixty to 80 percent of the Fe^{59}, which was lost from the circulating blood of the tumor-bearing animals in excess of that lost from the normal animals, was recovered in the tumor. In the animals bearing the solid tumors, only small amounts of Fe^{59} were found in the liver. In those animals bearing the Yoshida ascites tumor, however, an average of 15 percent of the injected radioiron was recovered in the liver. The spleen of animals bearing Hepatoma 3683 actually had less Fe^{59} than that found in the spleen of the normal control animals.

The distribution of Fe^{59} in rats bearing the Harderian gland carcinoma 2226 and C3H mice bearing the spontaneous mammary carcinoma is presented in table 4. The loss of red cells from the circulation of these animals began at a later point in tumor growth and proceeded at a slower rate. It will be seen that in Carcinoma 2226 a high proportion of the counts lost from the blood stream is recovered in the tumor. In the normal animals 6.5 percent of the injected radioactivity was recovered as noncirculating iron in the carcass, and 1.4 percent was in the liver. In the tumor-bearing animals, the same amount was found in the carcass, 5.3 percent was in the liver, and only traces were in the spleen and kidneys. The amount of circulating iron which left the blood stream of the tumor-bearing animals exceeds that lost from the blood stream of the normal animals by 21 percent, and of this, 19 percent was found as non-

TABLE 4.—*Distribution of radioiron in tumor-bearing animals following injection of Fe^{59} labeled red cells*

Tissue	Carcinoma 2226		Spontaneous mammary carcinoma*	
	Percent of injected dose		Percent of injected dose	
	N	T	N	T
Blood	92. 5	71. 2	66	42
Tumor	—	19. 1	—	17
Liver	1. 4	5. 3	4	6
Spleen	0. 0	0. 5	1	3
Kidneys	0. 0	0. 4	2	5
Carcass	6. 5	6. 6	10	5
Total	100. 4	103. 1	83	78

*Thirty-two days after injection of tagged erythrocytes.

circulating iron in the tumor. Although the data for the spontaneous mammary tumor follow the same general pattern, the recovery of isotope was less satisfactory, presumably due to technical difficulties in the experiments on mice.

An attempt was then made to find tumors in which there was little or no hemorrhage or thrombosis to determine if there was still a loss of circulating erythrocytes. Mouse Hepatoma 134 and Fibrosarcoma HE8971 were injected with Fe^{59} labeled cells 1 day after tumor implantation and the concentration of the Fe^{59} activity was observed in the peripheral blood. After a suitable period of time, the animals were killed and the distribution of the Fe^{59} activity was determined. The results are presented in table 5.

The Hepatoma 134 (ascites) had a milky ascitic fluid until the 10th day following implantation. Often a 20 gm. mouse would develop 15 ml. of ascitic fluid before traces of hemoglobin were found. No anemia and only minimal loss of labeled cells developed in an animal bearing this tumor until after the 10th day. The distribution of Fe^{59} in the animals killed on the 12th day is shown in table 5. It will be seen that 19 percent more radioiron had left the circulating blood in the tumor-bearing animal than in the normal animal, and that 11 of the 19 percent was recovered as noncirculating radioiron in the tumor.

The mouse Fibrosarcoma HE8971 was selected as a solid tumor with little evidence of hemorrhage. The anemia and loss of labeled cells occurred in the late stages of tumor growth, and then to only a limited degree. The distribution of Fe^{59} (table 5) on the 15th day shows that in the tumor-bearing animal 12 percent more radioiron had left the circulating blood than in the normal animal. Of this, 8 percent was in the tumor. The rat Fibrosarcoma 4956 was another tumor that showed minimal anemia and loss of counts into the tumor, though the tumors weighed 26 percent of the body weight. It was of interest to note, in confirmation of our earlier observations, that these animals which had little or no loss of labeled cells and no anemia had few signs of cachexia until the late stages of tumor growth.

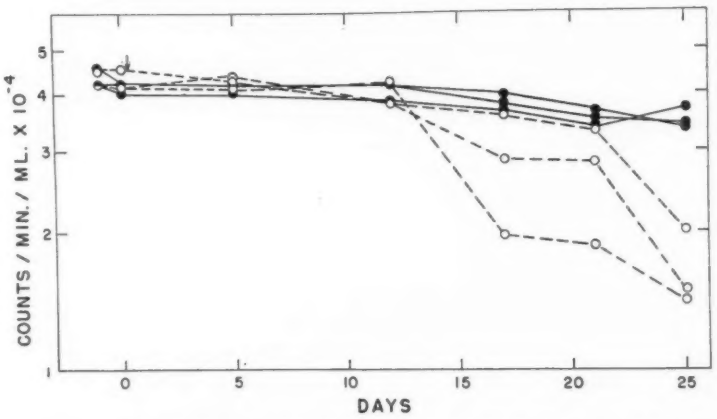
TABLE 5.—*Recovery of Fe^{59} following the injection of labeled red cells*

Tumor	Age of tumor (*days*)	Total weight/ body weight (*percent*)	Hemoglobin (*% of normal*)	Percent of injected Fe^{59} in blood		Percent of injected Fe^{59} in tumor
				N	T	
Hepatoma 134 (Ascites)	12	29*	76	83	64	11
Fibrosarcoma HE8971	15	12	93	91	79	8

*Total ascitic fluid containing 26 percent packed tumor cells.

Random Rate of Red Cell Loss in Individual Tumor-Bearing Animals

The studies of the previous section were carried out on groups of animals to lessen the problem of biological variation. In order to study the process in individual animals, Fe^{59} labeled cells were injected into 7 normal rats, 4 of which were injected subcutaneously 24 hours later with Lymphosarcoma R2788. When the animals were killed 25 days later, it was found that the blood volume and carcass weight had not varied significantly from that of the control animals, though the tumors averaged 10 percent of body weight. Therefore, any fall in the concentration of Fe^{59} in the peripheral blood which was greater than that of the normal controls should reflect the rate at which labeled cells had left the circulating blood. The curves for the individual rats are presented in text-figure 3. There



TEXT-FIGURE 3.—Fe^{59} counts in the peripheral blood of individual rats following the injection of labeled erythrocytes. ↓ = time of tumor implantation. ● = normal control animals. ○ = rats bearing Lymphosarcoma R2788.

was great variation from animal to animal in the rate at which the Fe^{59} left the blood stream and also marked fluctuation of the rate from day to day within the individual animal.

Distribution of Iron in Tumor-Bearing Animals

The data on the distribution of iron in the various organs of the rats bearing Lymphosarcoma R2788 and Hepatoma 3683 are given in table 6. In these studies, the amount of iron in the circulating blood was obtained by multiplying the Fe content per ml. of blood by the final blood volume determined by the injection of chromium-tagged red cells 30 minutes before the animals were killed. The iron values of the organs were obtained by subtracting from the total iron the amount of iron arising from the circulating blood—calculated by means of the following formula: Cr^{51} content of the organ $\times$ ratio of the concentrations of Fe/Cr^{51} in the blood = amount of circulating Fe remaining in the organ.

It can be seen in table 6 that these tumors accumulated a large amount of iron, exceeding the total amount of iron found in the liver. It can also be seen that the accumulation of iron in the tumors was largely balanced by a decreased amount of hemoglobin iron in the blood. Qualitatively these findings confirm those reported by us previously (*41*) in which no attempt was made to correct the total iron for circulating hemoglobin iron remaining in the organs.

TABLE 6.—*Distribution of iron in normal and tumor-bearing rats**

Tissue	Lymphosarcoma R2788				Hepatoma 3683			
	(*μg./gm.*)		Total μg. per 100 gm. body weight		(*μg./gm.*)		Total μg. per 100 gm. body weight	
	N (2)	T (4)	N	T	N (2)	T (2)	N	T
Blood.....	440	350	2600	2000	440	260	2200	1500
Tumor.....	—	50	—	620	—	46	—	670
Liver.....	140	120	460	420	110	130	430	470
Total.....	—	—	3060	3040	—	—	2630	2640

*Iron determined by the use of orthophenanthroline following a wet-ash digestion (*19*). All values are corrected for the iron in the circulating hemoglobin as described in the text.

Discussion

The data presented show that some rat tumors contain a large amount of iron which is derived from red cells. The extent of this process varies greatly from tumor to tumor. Where the process is extensive it is accompanied by anemia and marked signs of cachexia, and the animal dies while the tumor is still relatively small. Where the process is slower, the tumors become larger, the onset of anemia occurs later, and the animals live a longer period of time. Where the process is nil, the tumors grow to very large size, frequently reaching one third to two thirds of the total body weight, and signs of anemia and cachexia appear very late. Because of the magnitude of the process in some tumor-bearing animals and its relationship to the cachexia of the tumor-bearing animal, it becomes of interest to explore the mechanism of the process in more detail.

The passage of iron from the erythrocyte into the tumor could readily occur by a simple hemorrhagic process. The random rate at which labeled cells left the blood stream in the individual animals would be compatible with multiple hemorrhages into the area of the tumor. On the other hand, the areas of hemorrhage visible within the tumor were quite small compared with the amount of blood being lost from the blood stream, so that while a hemorrhagic process would be a simple mechanism to explain the type of process occurring, it seems difficult to explain the quantity of blood being lost by this mechanism.

If one postulates a hemolytic process, even greater difficulties are encountered in explaining the passage of erythrocyte iron into the tumor. If a hemolytic process were occurring, the damaged erythrocytes might either lyse immediately or be picked up by the reticuloendothelial system and destroyed. If lysis occurred immediately within the blood stream, one would have to postulate that the free hemoglobin in the plasma was picked up rather efficiently by the tumor, possibly as a source of nitrogen. Babson and Winnick (*54*) and Busch and Greene (*55*, *56*) have published data suggesting that the proteins of the plasma may serve as an important source of tumor nitrogen, and hemoglobin might be contributing to this pool.

On the other hand, if the erythrocytes were picked up and destroyed by the reticuloendothelial system, one would expect that a fairly large proportion of the radioiron of the labeled cells would be deposited in the spleen and possibly the liver. Actually, except for the Yoshida ascites tumor, relatively little of the Fe^{59} was found in the liver, and the amount in the spleen was essentially the same as that found in the normal controls. If any Fe^{59} from the erythrocytes phagocytized in the reticuloendothelial system was returned to the blood plasma, a major fraction would presumably be used for the synthesis of new red cells [8] to replace those lost by destruction. It therefore seems likely that if a hemolytic process is occurring, the passage of erythrocyte iron into the tumor would have to occur largely by way of direct intravascular hemolysis rather than through phagocytosis of the erythrocytes and passage of released iron through the blood plasma to the tumor.

The marked fluctuation in the rate at which the labeled cells are lost from the blood stream in individual animals would seem less likely to occur in a hemolytic process, particularly if a hemolytic factor was being released by the viable tumor cells. It could still be possible, however, if a hemolytic factor were being released intermittently from pockets of necrotic material. It should be noted that in Lymphosarcoma R2788, in which the process of erythrocyte destruction is most marked, relatively little necrotic material was found upon sectioning the tumor. In order to clarify the problem of whether a hemolytic or hemorrhagic process is occurring in these animals, further experiments elucidating the mechanism

[8] Unpublished experiments reveal a high rate of red cell synthesis and a marked reticulocytosis in these animals (*cf.* *59*, *57*).

of this process have been carried out and will be presented in the following paper of this series.

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Growth of Mouse Ascites Tumor Cells in Diffusion Chambers. I. Studies of Growth Rate of Cells and of the Rate of Entry of Antibody [1, 2]

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Summary

Sampling chambers, designed to permit the flow of ascites fluid but to exclude host cells, will admit isoantibody. Hemagglutinating, neutralizing, and protective effects have been demonstrated. The rate of fluid entry into cell-impermeable millipore chambers is slow, and the membrane acts as a considerable diffusion barrier to the entry of antibody. The peritoneum acts as an initial barrier to the flow of antibody from the blood stream to the chamber. Ascites tumor cells grow progressively in the sampling chamber after a sharp, early drop in cell number. The rate of rapid growth is sufficiently consistent to permit quantitative measurements of the effect of antibody.—J. Nat. Cancer Inst. 21: 657–670, 1958.

Cytotoxic effects of isoimmune serums against lymphoma cells can be demonstrated with a variety of techniques. The most precise is the *in vitro* method of Gorer and O'Gorman (*1*). For some purposes the combination *in vitro-in vivo* neutralization technique (*2*) or the demonstration of true passive immunity (*3*) has been widely used. The *in vitro* test tells little of conditions in the animal host; the *in vivo* methods cannot be used with any strict quantitation.

Algire, Weaver, and Prehn have developed the diffusion chamber and thereby made available a simple apparatus which can be used to give quantitative results in a controllable *in vivo* environment (*4, 5*). Nutrient and other substances can pass into or out of the chamber, and host cells can be either admitted or excluded by varying the pore size of the diffusion membrane. This flexibility has been exploited by the aforementioned workers who have shown that target cells placed in a cell-impermeable chamber can survive and proliferate even when placed in an immunized

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[3] We should like to acknowledge the help given to us, especially in the early stages of this investigation, by the late Dr. Glenn H. Algire, and to record our appreciation of the stimulus he gave to the study of tissue transplantation by his work and by the development of the many types of diffusion chambers in which he played such a large part.

homologous host. If appropriate immune cells are introduced, or if the pore size is increased to permit the passage of cells, the target cells are destroyed (*6*).

Falconer Smith, quoted by Algire *et al.* (*7*), has shown that heteroantibodies can enter the chamber, but in a somewhat lower concentration than is found in serum; such antibodies can destroy HeLa cells (*8*). By analogy, it seemed probable that isoantibody could also diffuse through the membrane. If so, the negative results obtained by these workers concerning the action of isoantibody must be reconciled with observations from other laboratories on the cytotoxic effect (*see 9*).

Several factors are responsible for the varying results. Some cells are sensitive to the action of serum while others are considerably more resistant (*9*). The relative concentration of antibody to cells is critical, and the physical state of the cells can determine whether antibody can reach the majority of the population or not.

For these reasons it was decided to study the properties of ascites cells in diffusion chambers, with especial emphasis on the lymphomas which, as a group, are known to be antibody sensitive (*10*). Before proceeding with cytotoxicity tests, it was necessary to study the growth rate of the tumors, the state of dispersion of the cells, and the ease with which isoantibody could enter the chamber. The results of these studies are presented in this paper.

Materials and Methods

Sampling chambers, of the type devised by Algire (*7,11*), made of lucite were generally used, but a few were of pyrex glass with the edges ground to provide a roughened surface for the cement. The chamber consisted of a cylinder some 16 mm. in diameter and 5 mm. deep. In all experiments (except those to determine the rate of fluid entry) an access hole large enough to admit a 22-gauge needle was drilled into the wall. The top and bottom of the cylinder were closed by discs of Millipore filter sealed with a cement made by dissolving millipore in acetone or with Acriloid cement.[4] The chambers were sterilized by leaving them in a 2.5 liter desiccator with 1.9 ml. ethylene oxide overnight. They were then left in free contact with air, usually for at least 24 hours, to allow the ethylene oxide to diffuse away. After the chamber had been filled, the access hole was closed by a tapered lucite peg, or in early experiments by a small square of millipore, either of which was glued in place with millipore cement. The glass cylinders have the advantage that they can be autoclaved after assembly but they are considerably more expensive.

Membrane.—The HA Millipore filter of average pore size 0.45μ was used for most of the experiments. For a few experiments a coarser cell-passing filter, AA, with a pore size of 0.80μ was used, and for some electrophoresis experiments extremely fine filters were used: VF, 10 mμ; VM, 50 mμ; and VC, 100 mμ.

[4] Acriloid cement is made by Rohm and Haas; its use was recommended to us by the Millipore Corporation Watertown, Mass., whom we also wish to thank for samples of Millipore filter.

Chamber procedure.—A known volume of a counted suspension of ascites cells was introduced into the sterile chamber through the access hole, which was then sealed. Recipient mice of appropriate genotype were anesthetized by intramuscular injection of sodium pentobarbital (Nembutal®). The Nembutal dose was varied according to the approximate size of the animal, from 1.5 mg. for a small mouse (approx. 20 gm.) to 2.5 mg. for a large (35 gm.) mouse. The skin was shaved, the abdominal cavity opened, and the chamber inserted, usually on the right side. The peritoneum was closed with a continuous silk suture, and the skin with 11 mm. Michel clips.

Sampling.—The abdomen was opened, the chamber removed, and the outside carefully swabbed with a pledget of cotton dipped in Ringer's solution or saline. Two holes were made in the membrane with a syringe needle, and the fluid contents aspirated with a tuberculin syringe. The edge of one layer of membrane was then cut around with the point of the needle and removed. Any fluid remaining could then be aspirated.

Frequently a clot was found in the chamber. The clots were usually small and filmy from the 2nd to the 4th days but might be more substantial on the 5th and 6th days. After this they might lyse, but this depended upon the tumor used and the degree of cellularity reached. The most dense clots were formed with leukosis E.L.4, the least with MC1M, which, however, produced quantities of hyaluronic acid, while the Ehrlich ascites clone E2 generally produced no clot.

Clots were usually minced *in situ* with the point of the syringe needle, washed repeatedly with Ringer's solution, and the expressed fluid was collected. In some experiments the clot was lysed with streptokinase-streptodornase, trypsin, or plasmin, the fluid collected, and the liberated cells were added to the rest of the chamber fluid. The lysis procedures were inconvenient for routine use, and it was found that the total number of cells collected were approximately the same if the minced clot was triturated in successive washes of saline. Sections of the fragmented clot usually showed relatively few cells trapped by the fibrin. It was estimated that only about 3 percent of the total cell population remained trapped in the clot.

A confirmation of these findings was obtained by plotting the growth curves of some tumors that had no clot in the early and late samples. The curve was not altered by the values obtained from chambers that contained a clot. Finally, some experiments were performed in which streptokinase-streptodornase was injected directly into the animal. The counts obtained from chambers in these animals were no higher and lower in some cases were lower than those in control mice.

Although it is possible to remove the tapered plug, sample the contents, seal, and replace the chamber, this was seldom done because the cell dispersion in the presence of a clot was inadequate for quantitative estimation.

Mice.—DBA/1 and C57BL mice were from the Roswell Park Memorial Institute colony maintained by Dr. Edwin Mirand; the others were from

Dr. Hauschka's colony. The origins of these mice have been described (*12*).

Tumors.—The Dalton DBA/2 lymphosarcoma has been used for the majority of experiments. Other tumors used were: DL1, a spontaneous lymphoma in DBA/2; the C3H lymphosarcoma 6C3HED and its DBA/2 adapted derivative, 6C3HED/2; the C3H sarcoma MC1M; the C57BL leukemias E.L.4 and E.L.24; the hyperdiploid Ehrlich ascites carcinoma; E2, a clone derived by Hauschka from the tetraploid Ehrlich ascites carcinoma; and QL1, a histiocytoma in DBA/1.

Tumor suspensions.—The tumors used in this work were all in the ascites form. Fresh ascites cells were diluted in a mixture of 10 percent mouse serum in mammalian Ringer's solution (NaCl, 8 gm., KCl 0.2 gm., $CaCl_2 \cdot 2H_2O$ 0.2 gm./liter) or in Krebs Ringer Robinson Kaltenbach [5] medium without serum, a dilution medium used successfully for clonal isolation (*13*). The growth rates were similar in both media, and clotting was a little less in the K.R.R.K.

The cell suspension was adjusted to the required concentration and kept on ice. It was always used within 3 hours and usually within 1 hour, depending on the number of chambers to be inserted.

Antibodies.—Isoantibodies were used exclusively. The immunizing procedures have been detailed elsewhere (*3*). Serums were obtained 10 days after 3 or 4 immunizing injections, tested for hemagglutinin titers (*14*), and then stored in the frozen or lyophilized state at −50° C. until used.

Results

Passage of Fluid into Empty Chambers

To determine the rate at which peritoneal fluid and therefore, presumably, antibody could enter, empty chambers were placed in mice of several strains, either immunized or normal. Most of the chambers were made up with cell-impermeable HA filters: AA filters, permitting cell entry, were used in a few instances.

The rate of filling was approximately the same for AA and HA filters, and for immune and nonimmune animals, and was remarkably constant. After a lag phase of approximately 6 hours, filling proceeded at the rate of 0.085 ml. per day until the chamber was almost full, usually in about 7 days. The walls of chambers removed during the filling phase were concave and air entered when the walls were punctured, whereas chambers placed in a beaker of saline retained their shape and remained empty after 7 days, indicating that filling was not a passive process but probably depended upon solution of air trapped in the chambers.

The rate of filling for chambers containing cell suspensions was more variable, the lag phase being longer and often considerably so. The rate of filling was never faster than that of the empty chambers.

[5] K.R.R.K. = Krebs Ringer Robinson Kaltenbach medium: NaCl 0.82 percent 710.0 ml., KCl 1.14 percent 31.0 ml., $MgSO_4.7H_2O$ 3.82 percent 8.0 ml., PO_4 buffer M/15 pH 7.2 46.0 ml.; $CaCl_2$ 1.22 percent 10.0 ml., glucose 4.5 gm., gelatin 1.25 gm., K ATP 0.120 gm., nicotinamide 0.300 gm. (N. B. Kaltenbach's original formula included 2.25 gm. gelatin.)

Passage of Antibody into Empty Chambers

Equilibrium in actively immunized mice.—In one typical experiment DBA/1 mice were immunized against the tumor DL1 by a course of 3 injections over a period of 2 months. Ten days after the last injection, when the blood hemagglutinin had reached a high level, 3 sets of HA sampling chambers were introduced into mice at 2-day intervals. All chambers were then removed, so that some chambers had been in place for 6, 4, or 2 days. The animals were killed, and samples of serum, peritoneal fluid, and chamber fluid taken. Hemagglutinin titers were measured. In every case there was a high level of hemagglutinin inside the chamber (table 1). The chamber fluid was approximately at the same level as the peritoneal fluid, and was either approximately at the same or occasionally at a slightly lower level than the serum. Essentially the same results on several occasions have been obtained with different strains and with somewhat different experimental details.

TABLE 1.—*Hemagglutinin titers in mice actively immunized against DBA/2 lymphoma**

Interval (*days*)	Serum	Peritoneal fluid	Chamber fluid
2	256–512	512	128–256
4	512–1024	256–512	256–512
6	512	256	256

*Titers expressed as reciprocal.

Since hemagglutinins could pass into the chamber, experiments were performed to see whether neutralizing activity could also be demonstrated and whether the chamber fluid could be used to transmit passive immunity.

Empty chambers were placed in DBA/1 mice immunized with a course of 3 injections of DL1 and left in place for 10 days. Upon removal, the hemagglutinin titer of the fluid in the chamber was found to be 1/512 for DBA/2 cells. One and a half ml. of chamber fluid was mixed with 630,000 DL1 cells in 1.5 ml. Ringer's solution and 0.2 ml. aliquots of the mixture injected into DBA/2 mice. Controls of DL1 cells in normal serum were set up. The control animals all developed tumors within 11 days; the experimental animals all remained tumor-free for over 15 days, though all ultimately developed tumors.

A similar experiment was performed in DBA/1 mice hyperimmunized against the DBA/2 lymphoma. Fluid from chambers that had been in place for 2, 4, and 6 days was collected and the fluids for each day were pooled. One-tenth ml. of the serum pool was injected into the abdominal cavity of a number of DBA/1 mice, which were challenged 24 hours later with 270,000 DBA/2 lymphoma cells subcutaneously. The controls all developed tumors within 8 days. The pooled fluid from each of the sets of chambers had protective effects, although not quite as good as that afforded by immune serum from the animals carrying the chambers.

Rate of fluid entry and antibody equilibrium in chambers borne by passively immunized mice.—Although it was clear that over a period of time the antibody concentration inside the chamber reached appreciable levels, the membranes limited the rate of fluid flow. It was possible that the diffusion rate of antibody was so slow as to prevent an adequate build-up in the presence of antigen—live cells might be capable of absorbing antibody as it entered. To gain some idea of the diffusion characteristics of the membrane, chambers were placed in the abdominal cavities of normal animals and allowed to become partially filled. After 2 to 4 days, when fluid was still entering the chamber, the mice were passively immunized with a known antibody intravenously, intramuscularly, or intraperitoneally. Simultaneous samples of chamber fluid, peritoneal fluid, and blood were then taken and compared for hemagglutinin content. When antibody was injected into the peritoneal cavity (care being taken to avoid puncturing the chamber) the level in the peritoneum was, of course, raised immediately; the level in the blood stream rose very rapidly usually reaching equilibrium within 30 minutes (*see also 3*), while little antibody penetrated the chambers within 30 minutes, although equilibrium was usually reached within 1 hour. There was some fluctuation between the results obtained in different experiments, and in some cases equilibrium was not reached until 90 minutes. Diffusion was considerably faster through an AA membrane that had larger pores.

When attempts were made to titrate antibody levels after serum was injected subcutaneously, intravenously, or intramuscularly, an unexpected delay was encountered. The titer in the peritoneal cavity remained very low for the duration of the experiments (2 to 3 hours) and antibody was not detectable inside any of the chambers (table 2). Antibody was always detectable after 24 hours.

As an extension of these experiments, chambers were placed in DBA/1 mice that were weakly immunized against a DBA/2 tumor. The chambers were left in place for 6 days. The serum-hemagglutinin level for DBA/2 cells was then 1/8 and the chamber fluid 1/4. Fresh antibody of

TABLE 2.—*Rate of entry of isoantibody into HA chambers*

Route of injection	Time after injection (*hours*)	Serum	Peritoneal fluid	Chamber fluid
Intraperitoneal	0	0	0	0
	½	256	2000	0
	1	512	256	64
	1½	256	512	64
	2	128	512	128
Intramuscular	½	32	—	0
	1	64	0	0
	1½	128	—	0
Intravenous	½	64	16	0
	1	128	32	0
	2	128	32	0
	3½	64	—	0

the same type but of much higher titer (1/1024) was then injected intramuscularly. The serum titer reached 1/32 after 30 minutes and 1/64 after 1 hour. The titer of the chamber fluid remained at 1/4 at the end of 30 minutes and had only reached 1/8 after an hour. It is clear that, while antibody can leave the peritoneal cavity freely, it enters this cavity from the blood stream much more slowly, and that the millipore presents an additional barrier.

Nature of the Chamber Fluid

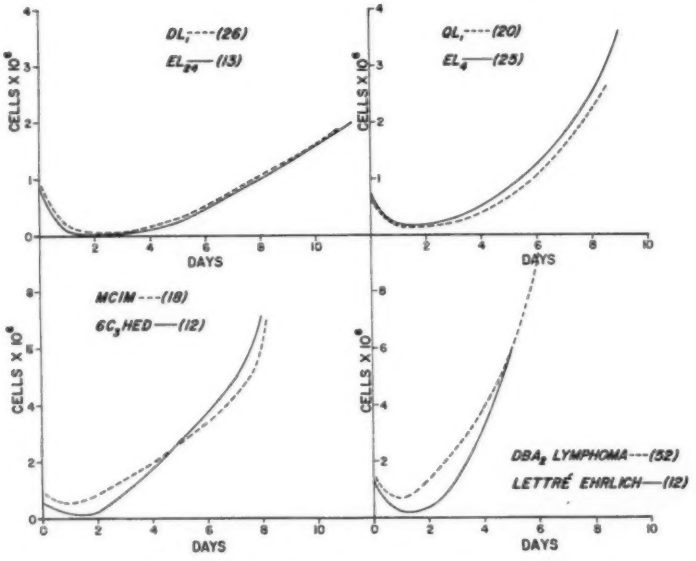
It has been reported by Algire and his colleagues that cells occasionally enter 150 μ HA chambers (*6*). We wished to learn how the different types of millipore varied in their ability to exclude not only host cells but also plasma proteins.

As anticipated, a large number of host cells entered the chamber through the coarse AA filters; they established themselves at a level of about 1,500 cells per mm.^{3}, and the chamber fluid was noticeably turbid. The fluids from HA chambers were usually of a clear, pale, straw color, and if the chamber had been *in situ* for more than about 3 days it contained a clot. In chambers inserted empty, the clots were usually small and friable; in some experiments with tumors, the clots were larger and relatively compact. In chambers sealed with millipore in acetone, there were usually no host cells. Occasionally as many as 15 per mm.^{3} were encountered; this is not ascribed to flaws in the membrane itself but to the inadequacy of the acetone-millipore cement. Since using Acriloid seal, breaches admitting host cells to the chamber have become exceptional. Faulty chambers, or those damaged by manipulation, were always discarded. The 2 or 3 cells per mm.^{3} found in earlier experiments can only have a negligible effect on the large target-cell population inside the chamber; but it is best to avoid this complication by the use of more efficient adhesives.

Electrophoresis was carried out in collaboration with Drs. Pinkel and Stickler (*15*). Samples were collected from chambers made with VF, VC, and VM filters as well as with HA and AA. With millipore in acetone as a cement, there was no difference in the electrophoretic pattern with any of these filters, although the VF is stated to have an average pore size of only 10 mμ. In experiments in hamsters performed by Pinkel, with Acriloid as a cement, fluids with extremely low globulin levels have been obtained. On the other hand, a high albumin and high fibrinogen level (source unknown) have been recorded on the supernatant chamber fluid from E2 ascites.

Growth Curves of Ascites Tumors in Chambers

Eleven ascites tumors have been grown in chambers. The growth curves of all except 6C3HED/2, which grew very slowly and stabilized at a very low cell concentration, showed similar characteristics; a sharp initial drop, followed in some cases by a low plateau and then by a rapid



TEXT-FIGURE 1.—The growth of 8 mouse ascites tumors in HA chambers. The number of determinations for each tumor is indicated in parentheses. All the tumors have been grown in the isologous strain, in addition, DL1, E.L.24, and the DBA/2 lymphoma have been grown in DBA/1; 6C3HED in C57BL; QL1 in 129 Rr; E.L.4 in 129 Rr and C3Hf/He. The hyperdiploid (Lettré) Ehrlich was grown only in Ha-ICR Swiss.

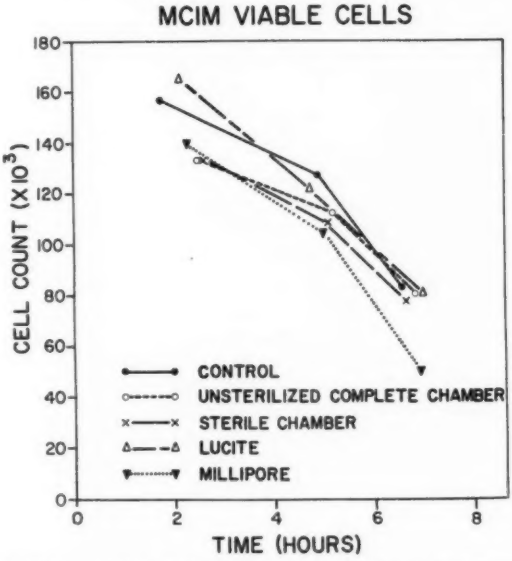
rise. No consistent differences in growth rate have been observed between a tumor grown in the isologous strain or in mice of a different genotype, although most of the tumors elicit a sharp immune response when injected directly into the peritoneum of a foreign strain. Some typical growth curves are given in text-figure 1, each representing the sum of a number of separate experiments.

Each tumor followed a characteristic growth curve, the most variable portion being the initial decline. Intermitotic times can be calculated from the slopes of the rapid growth phases and give reasonably consistent results for the same tumor. The slope of the curve on a semilog plot soon falls short of a straight line, indicating that the maximum rate of growth is not maintained for long.

The initial drop in cell concentration has always been encountered, not only with ascites tumors but also in experiments in which fragments or suspensions of spleen and of solid tumors were used. A number of experiments were set up in an attempt to modify or abolish the initial fall. Changes in the volume of suspending fluid, in the adhesive used for sealing, in the sterilizing procedure, and in the material used for the chamber cylinder had little effect. Normal serum or ascites supernatant from mice of the same strain as the tumor was no better as a cell suspending medium than pooled serum or K.R.R.K. medium alone. Alterations in

the initial cell count within the range tested, 20,000 to 2,000,000, affected only the lag phase but not the slope of rapid growth. In one series, cells were taken from the rapid phase of growth of the MC1M ascites and immediately transferred to a fresh chamber without dilution. Although the cells were in their steepest growth phase and were transferred in the same fluid in which they were growing, the initial fall was repeated.

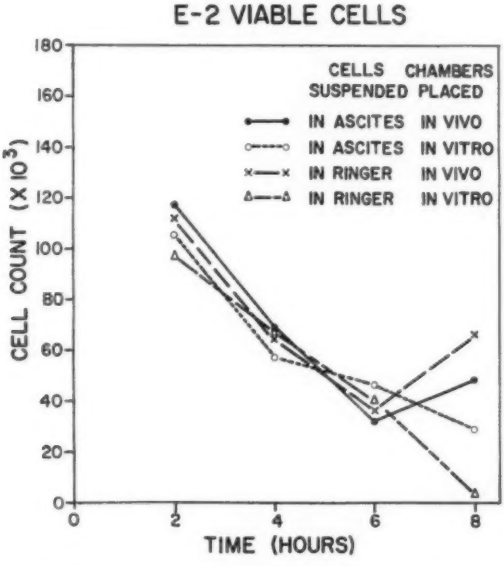
Révész and Klein found that ascites cells transplanted to the peritoneum grew to give a straight line in a semilog plot (*16*). Since our cells were growing in approximately this manner after the initial drop, we wondered whether this typical fall was due to toxicity in one or more of the components of the chamber. Accordingly, various chamber components were placed in flat-bottomed glass vials together with known cell suspensions in either Ringer's solution or in a 50 percent dilution of ascites fluid in mammalian Ringer's solution. The chamber components were: the lucite ring; 2 sheets of millipore; an intact, freshly glued chamber that had not been sterilized; and a complete freshly sterilized chamber. The walls of the complete chambers were broken to allow the free entry of cells. The tubes were then incubated at 37° C. and samples taken at 2-hour intervals after very thorough mixing. Duplicate or triplicate cell counts were made in a hemocytometer, and viable cells were estimated by counting the percentage of cells staining after dilution with 1/2000 trypan blue. As was to be expected, cell death was somewhat more rapid in Ringer's solution than in the diluted ascites, but no significant increase in the rate of destruction was found with any of the chamber components as compared to the controls (text-fig. 2).



TEXT-FIGURE 2.—A comparison between the numbers of viable cells after incubation in the presence of various components of the chambers.

As the experiments designed to detect toxicity of the chamber components had been performed *in vitro*, and because a number of other *in vitro* experiments had shown that ascites fluid or tissue-culture medium gave somewhat better survival than Ringer's solution, a final comparison was made between ascites fluid and Ringer's solution *in vitro* and *in vivo*.

A series of chambers was set up in 4 groups, 2 groups incubated *in vitro* and 2 *in vivo*. In the first group, chambers containing cells suspended in Ringer's solution were placed in a vial of Ringer's solution. In the second group, the fluid both inside and outside the chamber was ascites fluid diluted one-half with Ringer's solution. The third group of chambers contained cells suspended in Ringer's solution and placed in the abdominal cavity of Swiss mice. The fourth was similar to the third group but with ascites fluid used as a suspending medium. The viable cell count was essentially the same in each of the groups for the first 6 hours when the rate of fall in viable cells slowed down for the 2 intraperitoneal groups (text-fig. 3). Although ascites fluid, serum, and tissue-culture media containing serum gave better survival in some experiments than Ringer's solution *in vitro*, this did not appear to be the case *in vivo* where the protein content of the peritoneal fluid presumably performed the same function.



TEXT-FIGURE 3.—Number of viable cells after incubation of tumor cells *in vivo* or *in vitro*.

Discussion

The use of diffusion chambers for growth studies on ascites tumor cells permits a degree of quantitation that cannot be achieved in the intact animal in which infiltration of tumor cells and the little-studied cellular

response of the isologous host prejudice the accuracy of cell counts. Spread of tumors to the retroperitoneal and subcutaneous spaces and to the pleural cavity, to lymph nodes, and to viscera are too well known to warrant any further comment. The effects of this type of spread may be ignored in certain experiments where infiltration is minimal or the mass of the ascites tumor is great compared to that of the metastatic or local deposits.

An attempt to extend the experiments performed by Révész and Klein (*15*) on the growth of ascites cells *in vivo* to the use of smaller inoculums and to the first 24 hours of growth in the peritoneal cavity was only partly successful as the data cannot be interpreted unequivocally. There appears to be a cellular response even from a susceptible host, masking any changes in tumor-cell count of diploid lymphomas. In some experiments the number of host cells rose rapidly from about 4 to some 10 million. In others, with the readily identified MC1M or P288 cells, the same type of initial drop was found as occurs in the chambers, but the possibility that this was due to the migration of tumor cells into other organs or to the rinsing procedure could not be ignored. However, with larger inoculums, the fall was minimal or did not occur and early growth more nearly resembled that found by Révész and Klein.

In the chamber, quantitative measurements are handicapped by the occurrence of clots, by any adherence of cells to the millipore membrane and to the fall in cell numbers in the first 24 hours. These factors can be minimized by judicious choice of cell types. Adherence to the membrane is a problem with solid tumors which need some form of support for growth. Ascites tumor cells do not have this requirement, and sections of membranes, even after prolonged stay in the host, show only a few thousand tumor cells remaining on the millipore as compared to many millions in the clot or dispersed in the chamber fluid.

The clot presents a greater source of error, determined partly by the amount of fibrin laid down and partly by the cellularity of the chamber contents. The lytic methods tried were time consuming and the incubation required was sufficient to cause an error in any estimation of the proportion of viable cells. Trituration of the clot with repeated washes in Ringer's solution gave very high recovery; section of the remaining fragments of clot showed only a small proportion of cells left behind. The inside of the chamber was always carefully washed and all the washings were either counted separately or added to the original chamber fluid. We believe that the error due to cells being left behind in the clot or in the chamber is considerably less than 10 percent. The error will be minimized in comparisons between the results obtained on different days, or between chambers, and is certainly no greater than the errors in sampling and counting cell suspensions removed directly from the mouse peritoneum. For invasive tumors the error in the HA chamber technique is definitely smaller.

The cause of the initial fall in cell count is still not clear. It is probably due to the need for conditioning the medium rather than to toxic factors.

The process of conditioning the medium and possible molecular concentrations in the chamber against osmotic gradients is imperfectly understood. Investigators who have grown single or limited numbers of cells in culture (*17–19*) or who have potentiated the growth *in vivo* by a pabulum of damaged cells (*20*) have indicated how vital conditioning is for the growth of low concentrations of cells. It has been suggested that conditioning substances are diffusible (*21*) and experiments are being set up with specially designed chambers to test this hypothesis.

The interesting finding of Puck (*22*) that the irradiated feeder layer can be omitted after a few transfer generations has little application to our experiments in which it is desirable to keep nutritional selection away from the original ascites population to a minimum. The test cells should be obtained from animal passage for each experiment rather than being given the opportunity to adapt to continuous chamber passage. By using passively transferred antibody and by confining growth measurements to the rapid growth phase, the initial fall can be ignored.

The entry of antibody into the chamber is determined by the concentration of antibody in the peritoneal fluid, unless there is unusually intimate contact between a richly vascular organ and the membrane; this sometimes happens when the liver adheres to the chamber. In the absence of peritoneal inflammation, the protein content of the peritoneal fluid is about half that of blood serum (*23*). Nitrogen determinations on chamber fluid show a similar reduction from the serum level (*7*). The antibody content of peritoneal fluid would also be expected to be somewhat lower than the serum level and this has been found in the majority of experiments. This may partly explain the observation that many tumors grow better in the abdominal cavity of resistant animals than in the subcutaneous site.

In contrast to the peritoneum, the millipore membrane acts as a simple diffusion barrier so that the equilibrium concentration of antibody will be the same in the chamber and in the peritoneal fluid. Attainment of equilibrium across the membrane takes some time. This has been shown for changes in oxygen tension (*24*) as well as for isoantibody. Rise in oxygen tension in the peritoneal cavity is almost instantaneous when the animal is allowed to breathe pure oxygen. The rise inside the chamber takes some 5 minutes for completion. The antibody molecule is larger and equilibration takes considerably longer. This must be taken into account in any immunity experiment where absorption of antibody by cells will tend to upset the state of equilibrium unless the concentration of antibody is large compared to the target cell mass.

The results obtained to date suggest that the chamber-bearing animals should be passively rather than actively immunized so that both the time of administration and the antibody dosage can be regulated. A series of experiments to determine the effect of passively transferred antibody on ascites tumor cells is in progress and will be reported separately.

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Serum Enzymes. II. Glycolytic Enzymes in Patients with Cancer and Other Diseases [1, 2]

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Summary

Abnormal activity of several glycolytic enzymes was found in the serums of many patients with metastatic cancer. Of the enzymes measured, lactic dehydrogenase activity was most often abnormal. Most cancer patients in whom abnormal serum enzyme activities were found were also shown to excrete abnormally large amounts of creatine in the urine. Of conditions commonly associated with creatinuria, such as muscular dystrophy, dermatomyositis, pregnancy, thyrotoxicosis, malnutrition, and amputation, only muscular dystrophy and dermatomyositis were regularly accompanied by abnormal serum enzyme activity. Abnormal serum enzyme activities were frequently found in patients with recent myocardial infarction, hepatitis, nephritis, cerebral vascular accidents, and a variety of miscellaneous conditions not usually associated with creatinuria. The wide variety of conditions that were accompanied by abnormal serum activity of the various glycolytic enzymes only serve to emphasize the ubiquitousness of these enzymes in various body tissues and the lack of diagnostic specificity of abnormal serum activities of aldolase, phosphohexose isomerase, glutamic-oxalacetic transaminase, and lactic dehydrogenase.—**J. Nat. Cancer Inst. 21: 671–684, 1958.**

It is the purpose of this paper to present data on the activity of certain glycolytic enzymes in the serum of cancer patients. Warburg and Christian (*1*), in 1943, demonstrated abnormal activity of aldolase in the serum of rats with tumors. Since that time many groups have confirmed this abnormality in animals and man and have extended the observations to include other enzymes and other diseases (*2–40*).

While attempting to identify the factors responsible for serum enzyme abnormalities in patients with cancer, it seemed relevant to compare the

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activities of several serum enzymes in the same patient and to explore fully different disease states that might, or might not, be associated with abnormal serum enzyme activity. Of particular interest in this respect were diseases of the muscles, which had been found to be associated with abnormal activities of aldolase by Sibley and Lehninger (*25*) and by Schapira, Dreyfus, and Schapira (*23*). Since these muscle diseases are regularly associated with creatinuria, we were interested in measuring urinary creatine in cancer patients and in studying serum enzyme activity in patients with various diseases associated with creatinuria.

This paper details composite results of the comparative enzyme activities in the serum of patients with cancer and with a large number of different diseases. In a further paper (*41*) data will be presented on the physiological significance of serum enzyme abnormalities in patients with cancer, and, in particular, on our efforts to reverse the abnormalities and the consequences of such reversal.

Patients and Methods

Forty-eight normal subjects were volunteers who were recruited from different locations. Several normal children were studied to gain an approximation of normal values in children under 10 years of age.

One hundred and ten patients with cancer were studied. Each case had the diagnosis confirmed by biopsy, and many were reconfirmed at autopsy. Twenty-six patients with leukemia were tested. Bone-marrow aspiration was done in each case to verify the diagnosis.

A very large number of patients with a variety of other diseases were also subjected to repeated serum enzyme determinations. These patients exemplified the following diseases: myocardial infarction, primary muscle diseases, particularly muscular dystrophy, hepatitis, nephritis, pregnancy, thyrotoxicosis, malnutrition, and others. In all, 550 patients were examined.

The methods for drawing blood and preparing the serum samples have been described previously (*33*). It is worth re-emphasizing that the greatest care must be taken to avoid hemolysis in the specimen. The slightest visible hemolysis will release large amounts of the enzymes under consideration into the serum and invalidate any result.

The enzymes that have been measured are: aldolase, lactic dehydrogenase (LDH), phosphohexose isomerase, glutamic-oxalacetic transaminase (SGOT), and isocitric dehydrogenase (ICD). The assay methods for the first 4 enzymes have been described previously (*33*). Isocitric dehydrogenase determinations were made by the method of Wolfson and Williams-Ashman (*34, 35*). Urinary creatine measurements were performed by the Jaffé method.

In the present paper the expressions "serum enzymes" or "serum glycolytic enzymes" refer to lactic dehydrogenase, aldolase, phosphohexose isomerase, or isocitric dehydrogenase. No reference is made to proteolytic enzymes, amylase, lipase, phosphatases, or other unspecified enzymes.

Results

Normal values for the serum activity of the several enzymes measured in this study are reported in table 1. The mean, standard deviation, range and our arbitrary "upper limit of normal" are given for each enzyme. These values are in agreement with those in the literature.

TABLE 1.—*Normal values (48 adults)*

Aldolase (37 subjects)	
	4.67 units per ml. serum; S. D. 1.95 u.
	Range 1.2–9.3
	Upper limits of normal, 9.5 units per ml.
Phosphohexose isomerase (27 subjects)	
	17.6 units per ml. serum; S. D. 6.4 u.
	Range 5–33
	Upper limits of normal, 40 units per ml.
Lactic dehydrogenase (30 subjects)	
	80.4 units per 0.01 ml. serum; S. D. 11.6 u.
	Range 63–103
	Upper limit of normal, 110 units per 0.01 ml.
Glutamic-oxalacetic transaminase (23 subjects)	
	16.7 units per ml. serum; S. D. 3.9 u.
	Range 7.0–25.0
	Upper limit of normal, 32 units per ml.
Isocitric dehydrogenase (14 subjects)	
	123 mμmole per ml. serum; S. D. 52 mμmole per ml.
	Range 60–220 mμmole per ml.
	Upper limit of normal, 270 mμmole per ml.

Normal values for aldolase and lactic dehydrogenase activity in children are higher than in adults. We have taken the values reported by Hill for LDH (*12*) and by Schapira for aldolase (*23*) in children as probable normal levels, since both of these authors have studied this problem carefully with the technique employed here.

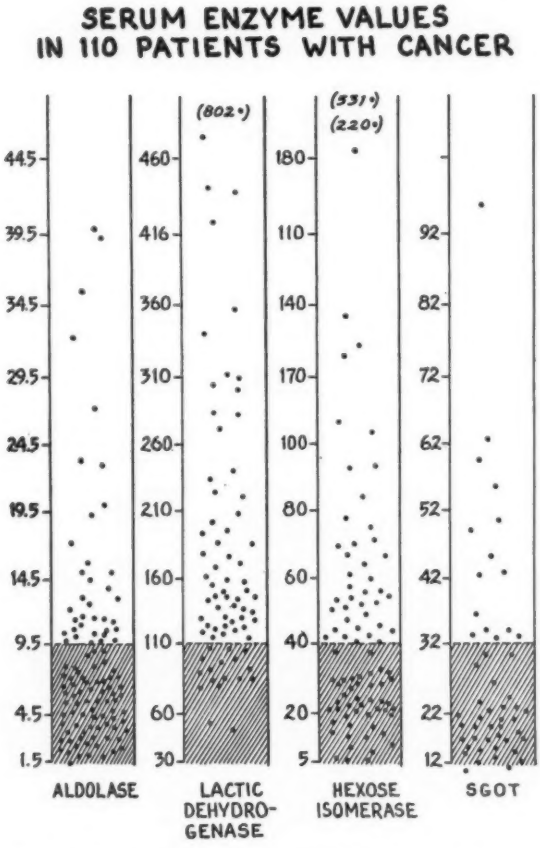
Serum enzyme activity in 110 patients with cancer showed remarkable variations, from low normal to markedly elevated values. With the arbitrary upper limit of normal, as defined, abnormal serum activity of the various enzymes was found as follows:

Aldolase—abnormal in 36 of 104 patients, or 35 percent
Phosphohexose isomerase—abnormal in 42 of 88 patients, or 48 percent
LDH—abnormal in 57 of 75 patients, or 76 percent.
SGOT—abnormal in 16 of 51 patients, or 31 percent.

It should be emphasized that the patients studied had many different types of tumors and represented all possible stages of the disease, from the earliest stages with no evidence of metastatic disease to a moribund status with masses of tumor. For this reason it did not seem feasible to present the actual values found in each case without presenting the clinical history. Text-figure 1 shows the variations of the several serum enzyme activities.

In this large group of cancer patients, various patterns of enzyme abnormality were seen. Certain patients had only a single abnormal enzyme value, usually an elevated LDH, while others had abnormal

activity of each of the enzymes measured. There was no apparent pattern associated with any type of tumor; rather the stage of the disease, regardless of site of origin, seemed more closely related to the enzyme activity in the serum. Most of the patients with abnormal values were in a late stage of the disease or actually in a terminal phase. Three types of tumor deserve special mention, however. Patients with multiple myeloma (of



TEXT-FIGURE 1.—Scattergram of serum enzyme values. *Shaded* area represents normal levels.

whom only 4 were studied) have regularly shown low values for each of the serum enzymes measured, regardless of the stage of the disease. However, such serums did not inhibit crystalline preparations of aldolase or lactic dehydrogenase; and one such patient, who developed infectious hepatitis, showed a marked elevation of aldolase and phosphohexose isomerase. Patients with reticulum-cell sarcoma involving nodes and patients with metastatic malignant melanoma had, with rare exceptions, markedly

elevated levels of glycolytic enzymes in their serums. The LDH activity of patients with reticulum-cell sarcoma was particularly high.

Although most of the patients had lost weight, profound wasting was not uniformly observed in those who had abnormal serum enzyme activity. One of the most malnourished patients in the group, who had obstructive carcinoma of the stomach, had normal serum enzyme activities serially for 7 months; conversely, a few patients with apparent good nutritional status were found to have high serum enzyme activities and marked creatinuria. Most of the patients with high serum enzyme activity had obviously widespread cancer.

Table 2 shows the serum enzyme activity, urinary creatine excretion, diagnosis, and clinical status of those patients with cancer in whom serial enzyme determinations were made. Urinary creatine determinations in 22 of the 26 patients shown in this table revealed abnormal amounts of creatine in all but 2. Both of these patients had normal activity of the enzymes measured. Four other patients excreted small amounts of creatine in their urine; 2 of these had near normal serum enzyme activity, while the other 2 showed considerable elevation of enzyme activity. The actual level of creatinuria correlated only roughly with the activity of aldolase in the serum, although high levels of both were found; the creatine concentration in the urine was more closely related to the activity of LDH. High LDH values were uniformly associated with high levels of urinary creatine.

Marked variability in the serum activity of the several glycolytic enzymes was found in 26 patients with leukemia (table 3). The highest values were found in patients with acute forms of the disease and only rarely in chronic lymphocytic or myelogenous leukemia. In patients with acute forms of leukemia, the serum activities of enzymes were high when the leukocyte counts were elevated, and normal or minimally elevated in those with low counts. Serial enzyme determinations in several patients with acute leukemia have shown fluctuations in the serum activity of LDH and aldolase, which correlates fairly well with the leukocyte count and less well with the activity of the disease itself.

Eighteen patients with the pseudohypertrophic form of progressive muscular dystrophy were examined and serial measurements of serum enzyme activity were made (table 4). No patient had normal aldolase or LDH values, although normal activities of phosphohexose isomerase and SGOT were found. The 4 patients with the lowest values for aldolase and LDH were the oldest patients in the group, with minimal activity of the disease. (All of these patients were less than 15 years of age. Normal values for the various serum enzyme activities have not been definitely ascertained. They are certainly higher than for adults.)

Eight patients with the facioscapulohumeral dystrophy of Landouzy-Déjerine were studied; just as the extent of the disease varied markedly in these patients, so did the activity of the serum enzymes.

Urinary creatine determinations in 6 of the patients with muscular dystrophy showed significant creatinuria in each (table 4).

Data on patients with a variety of other neuromuscular diseases, as

TABLE 2.—*Serum enzyme activity and urinary creatine excretion in patients with cancer*

Diagnosis	Stage	Aldolase (*u./ml.*)	LDH (*u./0.01 ml.*)	Phospho-hexose isomerase (*u./0.04 ml.*)	SGOT (*u./ml.*)	Creatine, urinary (*mg./24 hrs.*)
Carcinoma, tongue	Local spread	39. 7	130	130	55	180
Multiple myeloma (hepatitis)	Generalized	35. 6		106		495
Carcinoma, stomach	Widespread	32. 0		126		1783
Carcinoma, stomach	Liver metastases	27. 0	232	220	62	440
Carcinoma, lung	Widespread	23. 2	350	120		840
Carcinoma, bladder	Local spread	23. 1	434	90		310
Seminoma	Widespread	20. 0		83		75
Reticulum-cell sarcoma	Widespread	19. 4	370	136	34	664
Carcinoma, rectum	Local spread	17. 0		55		182
Reticulum-cell sarcoma	Widespread	14. 7	193	69	45	210
Carcinoma, lung	Widespread	13. 0	802	182		352
Melanoma	Widespread	13. 0		54		230
Carcinoma, stomach	Widespread	11. 5	203	47	55	873
Carcinoma, kidney	Widespread	10. 6		53		3335
Carcinoma, stomach	Localized	8. 0	98	44	33	
Reticulum-cell sarcoma	Widespread	7. 8	294	33	22	1420
Fibrosarcoma	Local spread	7. 3	279	44		363
Malignant schwannoma	Local spread	6. 6		43		100
Melanoma	Widespread	6. 4	223	48	42	193
Carcinoma, stomach	Localized	5. 2		27		5
Carcinoma, ovary	Widespread	4. 2	118	90	24	94
Rhabdomyosarcoma	Widespread	3. 3	116	26	24	60
Carcinoma, mouth	Localized	3. 2				0
Multiple myeloma	Widespread	3. 1	88	6	6	
Multiple myeloma	Widespread	2. 1	60			
Multiple myeloma	Widespread	1. 5				
Normal values		<9. 5	<110	<40	<32	

TABLE 3.—*Serum enzyme activity and leukocyte counts in patients with leukemia*

Patient	Diagnosis	Aldolase	LDH	Phospho-hexose iso-merase	SGOT	ICD	WBC per mm.³
Wien	Acute myel. leukemia	1. 9					<2, 000
Horn	Acute myel. leukemia	4. 2					<2, 000
Bass	Acute myel. leukemia	10. 8	164	34	12		>10, 000
Davi	Acute myel. leukemia	7. 8	148	34	30		?
Tuck	Acute myel. leukemia	10. 6	252	61	16		>50, 000
Meye	Acute myel. leukemia	12. 0	275	60	—		>40, 000
Smit	Acute myel. leukemia	7. 0	174	13	—		?
Rice	Acute myel. leukemia		272			286	
Hall	Subacute myel. leukemia	11. 3		37			>10, 000
Chri	Acute lym. leukemia	5. 3		20	22		<10, 000
Brid	Acute lym. leukemia	5. 7	154	7			>20, 000
Agee	Acute lym. leukemia		174			462	<5, 000
Tayl	Acute lym. leukemia	11. 4	57	20		671	17, 000
Hall	Acute lym. leukemia	3. 8	120	9	—		>20, 000
Brus	Acute mono. leukemia	10. 3	359	136			>20, 000
Cald	Acute mono. leukemia	7. 8	233	30			>40, 000
Lest	Chronic myel. leukemia	5. 4					<10, 000
Venn	Chronic myel. leukemia	5. 5	102	45			<10, 000
Bion	Chronic myel. leukemia	6. 0	119	38	—		15, 000
Kett	Chronic lym. leukemia	1. 9					<10, 000
Offu	Chronic lym. leukemia	4. 7					>10, 000
Osbo	Chronic lym. leukemia	4. 0	99	27	18		>50, 000
Pete	Chronic lym. leukemia	7. 0	95	23	10		>40, 000
John	Chronic lym. leukemia	4. 4	107	20	18		>30, 000
Hanb	Chronic lym. leukemia		114				>50, 000
Mart	Chronic lym. leukemia	10. 7	138	32		192	37, 500
Normal values		<9. 5	<110	<40	<32	<275	

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TABLE 4.—*Serum enzyme activity and urinary creatine excretion in patients with muscle diseases*

Patient	Diagnosis	Aldolase	LDH	Phospho-hexose iosmerase	SGOT	ICD	Urinary creatine (*mg./24 hrs.*)
	Pseudohypertrophic muscular dystrophy						
Rand		66. 8					
Buko		80. 4					
Sachs		23. 0	217	53	69		
Doug		91. 5	218	50	76	144	
Prue		23. 7	319	30	54	318	
Ques		11. 6	170	22	17		
Rice		28. 0	270	32	38	126	
Waeg		84. 6	260	63	101	132	
Codb		10. 7	145	17	18		625
Caob		12. 1	134	19	14		350
Gard		41. 5	338	71	190	228	
Rori		72. 0	574	59	125		140
Jori		21. 3	211	33	60		136
Russ		15. 2	454	18		108	
Shin		46. 4	402	84			
Swan		10. 8	160	6			
Frib			588			208	
Priw			658			288	
	Facioscapulohumeral dystrophy						
Bair		21. 2					
Nels		7. 6					
John		99. 4					
Scot		12. 0		20			1796
Ripp		6. 2		40			907
Hend		11. 5	86	11	35		
Brai		11. 2	91	25	30		
Morr		11. 7	95	21	35		
Normal values		<9. 5	<110	<40	<32	<275	

well as on patients with dystrophy, will be presented elsewhere. In general, acute inflammation of the muscles, trauma to the muscles and the various dystrophies are associated with marked abnormalities of serum glycolytic enzymes and constant creatinuria.

Serums from 139 patients with heart disease were submitted for enzyme analyses. These cases will be described in detail elsewhere. Seventy-eight of these patients had a clinical diagnosis of myocardial infarction. In 9 of these the infarction was old, *i.e.*, of more than 7 days' duration, yet a part of the episode leading to hospitalization. In these old cases abnormal activities of glutamic-oxalacetic transaminase, aldolase, and phosphohexose isomerase were not seen, and an abnormal LDH was uncommon. Abnormal serum LDH was constant in recent myocardial infarction, while abnormal SGOT values were found in only 50 percent of the cases of proved infarction. Table 5 lists our summary results.

TABLE 5.—*Patients with heart disease*

	(mean)	*(range)*
Recent proved myocardial infarction (69 patients)		
LDH in 68	245 u. per 0.01 ml.	115–795
SGOT in 51	55 u. per ml.	13–390
Aldolase in 36	13.3 u. per ml.	4. 3–50
Phosphohexose isomerase in 28	98.0 u. per ml.	23–690
Isocitric dehydrogenase in 11	194 mμmole per ml.	94–306
"Old" myocardial infarction (9 patients)		
LDH in 8	97 u. per 0.01 ml.	69–139
SGOT in 8	16.7 u. per ml.	9. 0–23. 5
Aldolase in 4	5.5 u. per ml.	1. 0–6. 7
Phosphohexose isomerase in 4	29 u. per ml.	24–33
Angina pectoris without proved infarction (61 patients)		
LDH in 59	93 u. per 0.01 ml.	41–137
SGOT in 40	16 u. per ml.	5–35
Aldolase in 23	6.6 u. per ml.	1. 1–15. 8
Phosphohexose isomerase in 27	27 u. per ml.	5–62

Fourteen patients with acute infectious hepatitis were studied with the serum enzyme battery. Abnormalities of LDH, aldolase, and glutamic-oxalacetic transaminase were seen in every patient examined during the first 2 weeks of illness. Marked elevation of the isocitric dehydrogenase activity of these serums was found (table 6). In the patients with hepatitis the ICD activity was invariably markedly abnormal, whereas, with each of the other enzymes, there were patients who had either no abnormality or minimal elevation of the serum activity.

In 15 other patients with various forms of liver or biliary-tract disease, irregular abnormalities of the several serum enzymes were found. Serum ICD was abnormal in every patient with cirrhosis in the active stage, while the other enzymes were only occasionally abnormal.

TABLE 6.—*Serum enzymes in patients with infectious hepatitis*

	Total number patients—14		
	Mean	Range	Normal range
Aldolase in 5	34.5 u. per ml.	7. 0–91. 6	1. 2–9. 3
LDH in 13	176 u. per 0.01 ml.	96–302	63–103
SGOT in 7	112.0 u. per ml.	10–166	7. 0–25. 0
ICD in 7	1197 mμmole per ml.	440–2750	60–220

An attempt was made to measure the serum enzyme activity in a group of patients with various conditions usually associated with creatinuria. Eleven pregnant women were examined. One of them had minimal elevation of the phosphohexose isomerase activity of her serum. No abnormal LDH values were found. Seven patients who had undergone midthigh amputations were studied. In none was an abnormal activity of any of these serum enzymes found. Two patients with anorexia nervosa and severe malnutrition had completely normal serum enzyme tests. One severely thyrotoxic patient had elevated activity of aldolase and phosphohexose isomerase. Two other similar patients, with an apparently comparable clinical status, had normal aldolase but elevated phosphohexose isomorase activity.

Eleven patients with renal disease were examined. Of these, 9 had glomerulonephritis. Serum LDH activity was abnormally elevated in each of these patients, while phosphohexose isomerase and aldolase activities were normal in all but one. There were no abnormal SGOT values. A single patient with acute renal failure had an elevated LDH assay; a single patient with unexplained proteinuria had normal LDH and SGOT activity. Similarly, of 6 patients with lupus erythematosus disseminatus, the only serum enzyme abnormality was the LDH, which was abnormal in 3 of the 6 patients.

Four patients with acquired hemolytic anemia had abnormal serum activity of aldolase, LDH, SGOT, and phosphohexose isomerase, confirming the fact that hemolysis can invalidate any serum enzyme determination.

Of a large group of patients with a variety of disorders including peptic ulcer, infectious mononucleosis, renal colic, hypoproteinemia, Gaucher's disease, and Paget's disease of the bone, only a few had abnormal serum enzyme values, and these could not be considered of diagnostic importance.

Discussion

It is apparent from the data that there is great variability in the activity of the several serum enzymes in patients with cancer. It must be stressed that no effort has been made to divide these patients by clinical status or histological type of tumor. For this reason there are, included in the group, patients with very small amounts of tumor and essentially no disability, as well as others with extensive metastatic disease and a mori-

bund status. To compare such patients in the same group is to ignore the very great differences that exist between different types of cancer or within the same histological type between an early and a late case.

A second reason for the great differences in the serum enzyme activities may have been the presence of liver metastases in many, but not all, of the patients studied. From the data shown in table 6, it is quite apparent that liver damage in patients with hepatitis may produce the most striking abnormalities of several of the serum enzymes. In many patients with cancer it is difficult to assess clinically the presence of liver metastases at an early stage. Serum alkaline-phosphatase levels may, in these cases, be of diagnostic significance if they are abnormal. Other serum enzymes may be altered in the presence of liver metastases (*39*). In such cases it would be difficult, if not impossible, to differentiate between the role of tumor metabolism itself and liver damage in the production of the enzyme abnormality.

In 22 patients with cancer, 24-hour urine creatine excretion was measured. Normal creatine output was found in a few patients who had normal serum enzyme levels. With two exceptions, patients with elevated serum enzyme values were found to excrete abnormal amounts of creatine.

Abnormal creatine excretion has been found by Benedict *et al.* (*42*) and by others (*43*) to be associated with decreased storage of creatine in the muscles in the presence of normal hepatic synthesis of the compound. Creatinuria is most dramatic in degenerative and inflammatory diseases of the muscles and is common in chronic muscle wasting or decrease in the muscle mass. In the latter regard it usually follows amputation of an extremity (*44*). Creatinuria is normal in children and pregnant women and occurs in a very minor degree in some mature women. It is virtually absent in mature men.

No abnormal enzyme activities were found in pregnant women, contrary to the report of Hill and Levi relative to LDH (*11*). No abnormalities were found in patients with profound malnutrition, a result that had already been described by Warburg (*1*). Normal serum enzyme assays were found in 7 patients with midthigh amputations studied at varying intervals after operation. One patient with thyrotoxicosis had abnormal amounts of glycolytic enzyme activity in her serum. The patients with muscular dystrophy and those with dermatomyositis showed the most pronounced abnormalities of all the enzymes measured, with the exception of isocitric dehydrogenase values, which were normal. Similar findings have been described by others (*11*, *20*, *21*, *23*, *25*).

The data reported on patients with myocardial infarction confirm those previously reported. It is of great interest that ICD activities were normal in 10 of 11 patients with recent myocardial infarction, the single exception being a patient with marked congestive failure complicating the infarct, in whom the ICD activity was just above normal. This failure of the ICD to rise in serum after myocardial infarction, at a time when LDH, aldolase, phosphohexose isomerase, and SGOT

values are high, is particularly striking because myocardium is rich in ICD (*45*). This discrepancy brings into sharp question the pleasantly simple notion that the serum enzyme abnormality which follows myocardial infarction is strictly a result of the outpouring of the enzyme contents of necrotic cells (*46*). It brings further into focus the great need for exact data on the permeability of cells to the enzymes contained therein, and on the half-life in serum and route of excretion of each of the enzymes under study.

The data on patients with liver disease confirm those of many other authors (*2, 3, 10, 14, 19, 28, 34–37, 40*). Of particular interest are the ICD activities in patients with hepatitis, which are in agreement with those reported by Wolfson *et al.* (*34, 35*). Abnormal serum activities of this enzyme appear to be found almost exclusively in the presence of liver damage, whether due to hepatocellular disease, biliary obstruction, or carcinoma.

The isolated abnormal values in diseases involving the kidneys, connective tissue, brain, lung, erythrocytes, and prostate serve to emphasize the widespread distribution of these enzymes in tissues, and the bewildering number of conditions which can be shown to be associated with abnormalities of serum glycolytic enzyme activity.

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Serum Enzymes. III. The Significance of Abnormalities of Glycolytic Enzymes in the Serum of Cancer Patients [1,2]

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Summary

The abnormal serum activity of aldolase, lactic dehydrogenase, phosphohexose isomerase, and glutamic-oxalacetic transaminase, often found in patients with cancer, may decrease to normal following the administration of large amounts of protein. In some patients with liver metastases, protein administration fails to lower the serum enzyme activity. Similar studies in patients with muscular dystrophy, all with high serum activity of these enzymes, do not demonstrate any effect of protein feeding on serum enzyme activity. In cancer patients the fall in serum enzyme activity after protein feeding is not a result of enzyme inhibition by albumin or Amigen. Protein feeding in some patients with cancer may be associated with an explosive increase in the apparent rate of tumor growth.—**J. Nat. Cancer Inst. 21: 685–696, 1958.**

It is the purpose of this paper to present data on the effect of dietary manipulation on the activity of certain glycolytic enzymes in the serum of patients with cancer.

Previous studies in this laboratory have shown that in patients with a wide variety of tumors there is frequently a close correlation of abnormal serum activity of aldolase, lactic dehydrogenase (LDH), and phosphohexose isomerase with the level of creatine excreted in the urine (*1*). Measurement of serum enzyme activity in patients with a number of diseases regularly associated with creatinuria has revealed that many such patients, particularly those with primary diseases of the muscles, have marked abnormalities of the serum activity of aldolase, LDH, and phosphohexose isomerase (*2–4, 1, 5*). It has been conclusively shown that in adults creatinuria is due to a decrease in muscle mass or to active muscle disease in the presence of normal hepatic synthesis of creatine (*6, 7*).

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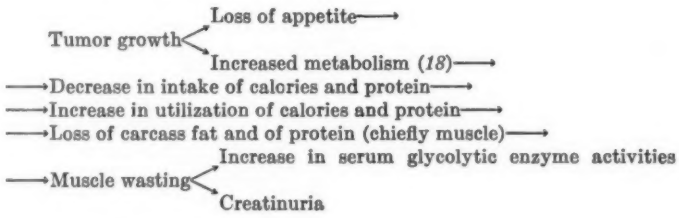
[3] With the technical assistance of Barbara Maxon.

One must assume that creatinuria in patients with cancer is related to muscle wasting just as it is in other diseases.

It has been abundantly demonstrated that tumors take up and hold amino acids tenaciously, even in the presence of severe starvation (*8–13*). Fenninger and Mider have shown that rats bearing certain tumors undergo considerable carcass wasting, particularly of the muscles (*14–16*). The tumors, however, show a continuous growth and increase in nitrogen content despite a decrease in food intake by the tumorous animal, and profound wasting of its non-tumor carcass (*14*, *15*, *17*).

In an attempt to explain the mechanism of production of the abnormal serum enzyme activity in cancer patients, almost all of whom have shown creatinuria, the observations of Zamecnik, LePage, Mider, Fenninger, and others seemed germane. A diagrammatic flow sheet of the effect of tumor growth appears as follows:


Loss of appetite
Tumor growth
Increased metabolism (18)
Decrease in intake of calories and protein
Increase in utilization of calories and protein
Loss of carcass fat and of protein (chiefly muscle)
Increase in serum glycolytic enzyme activities
Muscle wasting
Creatinuria

If this schematic representation were correct, it would follow that interruption of the chain of events at any stage prior to muscle wasting should prevent or correct creatinuria and the serum enzyme abnormality. Removal of the tumor could do so, as reported by Warburg (*19*) and by Sibley (*20–22*). At a later stage, by feeding very large amounts of protein and calories, one should be able to supply all the needs of the tumor and of the non-tumor carcass. Should such feeding be associated with a decline in the serum activity of the glycolytic enzymes and in creatinuria, it would be strong evidence that the abnormality was, indeed, secondary to inadequate protein supplies rather than a direct effect of the tumor.

The present report details the results of such dietary manipulations.

Patients and Methods

From a group of 110 patients with cancer on whom repeated serum enzyme determinations were performed (*1*), 22 were selected for intensive study. All but 1 had abnormal elevation of 1 or more serum glycolytic enzymes. In most of the patients, urinary creatine output was measured. Each patient was then placed on a diet chosen *ad libitum* but measured accurately for caloric, protein, fat, and carbohydrate content. While the patients were on such diets, serum enzyme activity, serum protein content, various liver-function tests, weight, and urinary creatine content were measured.

Serum aldolase (*21*), phosphohexose isomerase (*23*), lactic dehydrogenase (*24*), and glutamic-oxalacetic transaminase (SGOT) were measured by

methods in common use and previously described (*25*). Urinary creatine and creatinine were measured by the Jaffé method (*26*). The dietary intake was calculated, not directly measured, except in 2 patients who were on total metabolic balance studies in whom the intake as well as output was precisely determined.

Dietary manipulation was performed by simply adding eggnog supplements of high protein content. In those few patients for whom the diet could be supplemented orally, the protein intake was generally increased from approximately 50 to 100 gm. per day. Such supplements had the advantage of ease and of closer approximation to natural conditions but the strong disadvantage of depending on the desire and ability of the patient to take all the diet and eggnog. In most patients oral supplementation was not feasible, and intravenous alimentation was used. This was given in the form of Amigen® in amounts from 50 to 150 gm. daily.[4] In several experiments, the intravenous supplement was given in the form of human serum albumin.[5] In 3 studies the diet was supplemented with intravenous Lipomul® without any change in the protein intake.[6]

While intake of protein, fat, carbohydrate and the number of calories were being augmented, the various measurements were continued. Serial serum enzyme activity, urinary creatine and creatinine excretion per 24 hours, weight, and size of the tumor, either by direct measurement with calipers or indirectly by X ray, were recorded.

Results

Effect of protein feeding on serum enzymes.—Our experience in protein feeding of 22 patients with cancer is summarized in table 1. It can be seen that in 12 of 20 patients the feeding of large amounts of protein, either by mouth or, more commonly, by vein in the form of Amigen, resulted in a fall of serum enzyme values toward, and often to, normal activities. Those patients in whom such a fall was seen had several different types of tumors, and some had metastatic disease in the liver. Of the 7 patients who had no evidence of liver metastases either by physical examination or liver-function tests, 6 showed a fall to normal serum enzyme activity after oral or parenteral protein supplementation. The seventh patient died unexpectedly on the 2nd day of protein administration. In the patients with apparent liver metastases, the response of serum glycolytic enzymes to protein feeding was less predictable. In a few with initial marked elevation of one or more of the enzymes, a prompt fall in activities occurred with dietary additions. In others with presumably the same degree of liver involvement, protein feeding did not produce a fall to normal enzyme activities, though occasionally a decline was seen. In no patient was there significant change in weight or in hematocrit readings.

[4] The Amigen, which was generously supplied by Mead Johnson & Co. through the courtesy of Dr. Robert Little, contained less utilizable amino acid than the listed strength. Amounts recorded make allowance for this discrepancy.

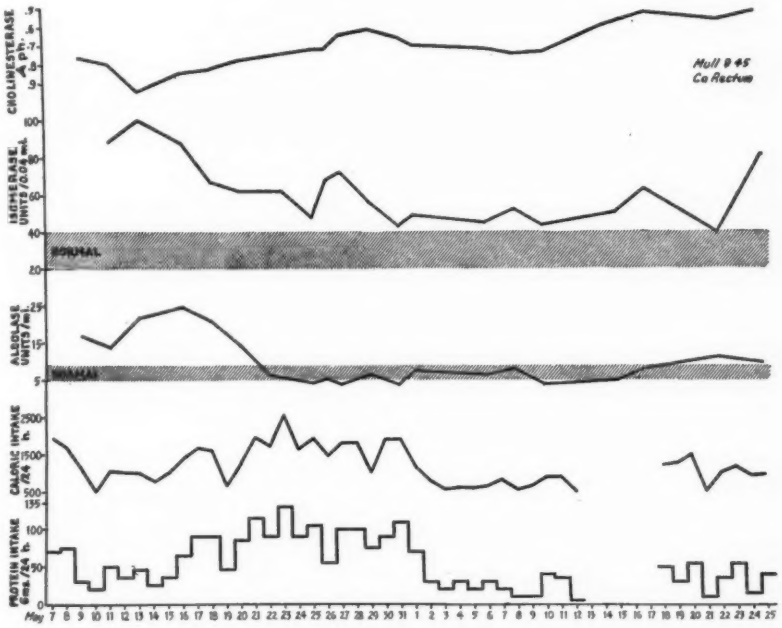
[5] The albumin was supplied through the generosity of the American Red Cross and the kind offices of Dr. Sam Gibson.

[6] The Lipomul was supplied by The Upjohn Company through the courtesy of Dr. E. A. Hawk.

TABLE 1.—*Effect of protein feeding on serum enzyme activity*

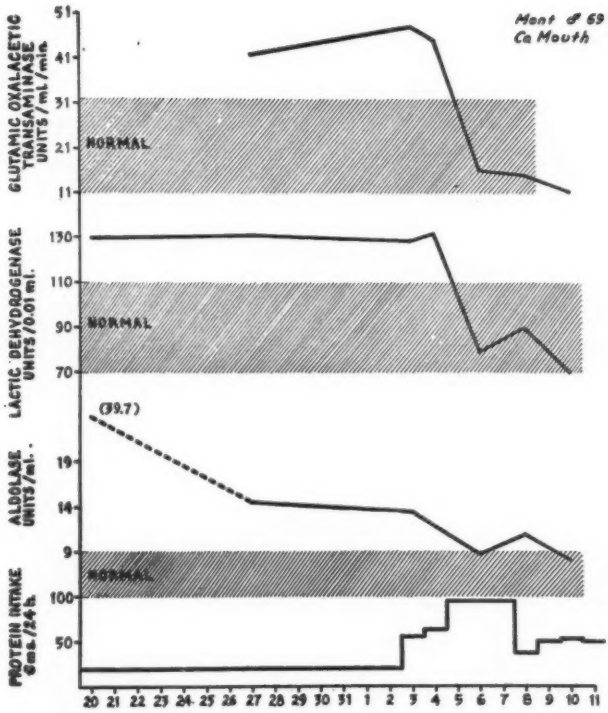
Patient	Diagnosis	Protein intake (*gm./day*)	Aldolase (*u./ml.*)		Phosphohexose isomerase (*u./ml.*)		LDH (*u./0.01 ml.*)		SGOT (*u./ml.*)		Urinary creatine (*mg./24 hours*)	
			Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
	With liver metastases											
McGi....	Lymphosarcoma.....	165(Amigen)	33. 9	18. 2	331	139	1640	1328	57	13	664	227
Brow....	Melanoma.....	50(HSA)	28. 0	12. 4	—	—	—	—	—	—	514	—
McGo...	Carcinoma stomach..	120(Amigen)	24. 3	37. 7	220	161	223	163	125	235	440	429
Dash....	Carcinoma lung.....	120(Amigen)	23. 2	27. 0	120	139	350	661	—	—	840	212
Trum....	Carcinoma lung.....	65(Amigen)	16. 0	6. 1								
Peil.....	Melanoma.....	100(Amigen)	15. 0	8. 1								
Harl.....	Lymphosarcoma.....	100(HSA)	14. 7	6. 3	69	—	193	223	45	45	210	297
Brow....	Carcinoma lung.....	100(HSA)	13. 0	7. 2	182	90	802	432	—	—	352	15
Shel.....	Melanoma.....	30(Lipomul).	13. 0	12. 0	54	75	—	—	—	—	230	250
Issi.....	Carcinoma lung.....	125(Amigen)	11. 5	21. 3	47	51	203	213	55	40	873	800
Sanm....	Lymphosarcoma	110(Amigen)	10. 4	11. 4	39	57	580	443	19	15	1420	—
Perk.....	Carcinoma ovary....	60(Amigen)	8. 5	3. 0	64	51	160	189	65	18	36	32
Wils.....	Carcinoma lung.....	70(HSA)	8. 1	3. 9	39	6	187	98	26	8	—	—
Morr....	Melanoma.....	50(HSA)	6. 4	6. 1	48	35	233	133	42	24	193	147
	Without liver metastases											
Mont....	Carcinoma tongue	90(Amigen)	39. 7	8. 1	—	—	130	78	42	11	180	10
Ahre....	Carcinoma bladder..	100(HSA)	23. 1	19. 0	90	72	434	445	—	—	310	125
Mull....	Carcinoma rectum...	120(oral)	17. 5	7. 6	85	45	—	—	—	—	182	149
Harr....	Carcinoma lung	140(oral+HSA)	10. 5	2. 8	—	—	—	—	—	—	245	330
Gayl....	Carcinoma tongue...	120(Amigen)	9. 9	8. 5	34	25	158	102	—	—	—	—
Vand....	Fibrosarcoma.....	110(Amigen)	7. 3	7. 2	44	28	279	110	—	—	363	?
Paul.....	Carcinoma breast....	55(HSA)	6. 1	4. 6	41	33	185	92	19	20	—	—
Powe....	Carcinoma stomach..	0	5. 2	5. 0	27	16	—	—	—	—	7	0
Normal range.....			1. 2–9. 3		5–33		63–103		7–25			

Text-figure 1 demonstrates the sequence of events in a patient with carcinoma of the rectum, with local recurrence within the pelvis and no evidence of liver metastases. For the first 8 days, while the patient was on an *ad libitum* diet, serum aldolase and isomerase activities fluctuated slightly at elevated figures. On the 9th day, the oral protein intake was increased from an average of 35 gm. daily to 90 gm. daily, with a modest concomitant rise in caloric intake. There was a prompt fall in aldolase to normal and of isomerase almost to normal. There was no change in creatinuria during the period of the experiment. Later, when protein and caloric intake had dropped, there was a second rise of both aldolase and isomerase.



TEXT-FIGURE 1.—Chart of a 45-year-old woman with carcinoma of the rectum. This patient had massive local recurrence without clinical or laboratory evidence of liver metastases. Cholinesterase values included as 1 test of hepatocellular integrity.

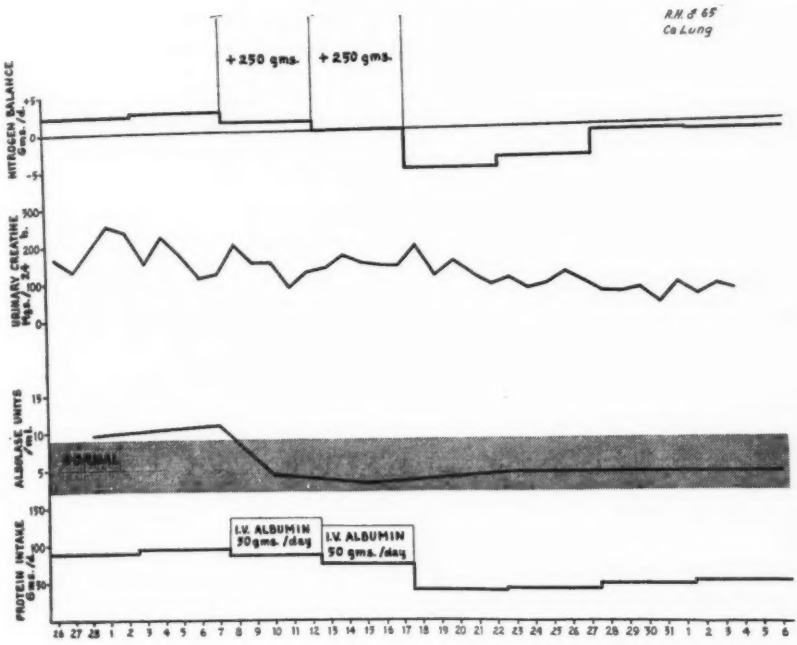
Text-figure 2 demonstrates a similar response in a patient with carcinoma of the floor of the mouth, with local invasion of the base of the tongue, and metastases to cervical and supraclavicular lymph nodes. This patient had existed for about 80 days on a liquid diet, which he swallowed with great difficulty. His protein intake during that period averaged 20 gm. daily. Serum activity of aldolase, lactic dehydrogenase, and glutamic-oxalacetic transaminase were high during this period. Four days after the intravenous infusion of Amigen was begun in amounts of 50 to 100 gm. daily, the serum activity of each of these enzymes was



TEXT-FIGURE 2.—Demonstrates effect of intravenous Amigen on serum enzyme activities in a patient with tumor limited to head and neck.

normal. There was a slight secondary rise in aldolase, which then again fell below normal. During the Amigen infusion the tumor, which had very gradually enlarged over the previous 3 months, appeared to gain a tremendous spurt of growth and enlarged dramatically. On the 9th day after Amigen infusion was started, the patient suddenly expired.

Text-figure 3 demonstrates the effect of human serum albumin on the aldolase content of serum. This patient, who had carcinoma of the lung with mediastinal metastases but without apparent liver metastases, had a positive nitrogen balance on a diet of 90 gm. of protein daily. Despite this externally positive balance of nitrogen he had significant creatinuria and elevated serum aldolase activities on several successive determinations. The intravenous infusion of 500 gm. of human serum albumin over a 10-day period, in addition to ensuring a markedly positive nitrogen balance, was associated with a prompt fall of serum aldolase to low values and a gradual fall of urinary creatine excretion to negligible quantities over the next 2 weeks. Clinically, his condition deteriorated during the albumin administration, and at the end of the experimental period he had developed superior vena caval obstruction, which was treated with X ray 1 week after completion of this study.



TEXT-FIGURE 3.—Effect of human serum albumin on aldolase content of serum. This patient, in positive nitrogen balance on an intake of 90 gm. of protein daily, had creatinuria and elevation of serum aldolase of modest degree. Administration of 500 gm. of human serum albumin was promptly followed by a fall to low activities of aldolase and a gradual fall of urinary creatine excretion. Simultaneously his mediastinal tumor enlarged.

Seven other patients received intravenous human serum albumin as the source of increased protein intake. Three of these patients showed a rapid decline of all abnormal enzyme values to normal following intravenous administration of the albumin. In 3 other patients the serum activity of one enzyme declined without change in the others. In 1 patient aldolase values fell toward, but never to, normal. One patient showed no change in any of the enzymes measured. Similarly, 5 patients who received large amounts of Amigen intravenously showed no fall in the serum enzyme activity. One patient who received intravenous fat evidenced no change in the activity of aldolase or isomerase in the serum.

One patient deserves special mention. This man, who had an obstructive carcinoma of the stomach without obvious liver metastases, was maintained on total parenteral alimentation for 7 months. During this time, he had serial serum enzyme determinations while he was receiving many different levels of caloric and protein intake. At no time during his entire course of treatment was an abnormal serum enzyme value detected, despite the most profound wasting and almost constant fall in urinary creatinine excretion and short periods of creatinuria.

Effect of protein feeding on symptoms.—Most of the patients in this group were anorectic to a greater or lesser degree. There was little obvious effect on appetite from the administration of Amigen, Lipomul, or human serum albumin. In some patients the protein hydrolysate seemed to aggrevate nausea, but in most it had no such effect. A few patients felt markedly better after intravenous feeding, with return of strength and well-being and improvement of appetite. Such improvement was, in a few, accompanied by an obvious increase in tumor mass. Most patients noticed little if any effect on their symptoms.

Effect of protein feeding on tumor growth.—In several patients there was an apparent acceleration in the rate of tumor growth following the protein supplementation, whether by mouth or by vein. This was most striking in 3 patients in whom subcutaneous tumor was palpable; in 1 patient cervical metastases from carcinoma of the mouth nearly doubled in size in 8 days, whereas previously they had barely grown in 3 months. In most of the patients the position of the tumor or an inadequate observation period prior to dietary manipulation made it impossible to evaluate the effect of high protein intake on tumor growth in any objective way.

Effect of protein supplements in serum enzyme activity in patients with muscular dystrophy.—To study the effect of large amounts of Amigen or albumin in patients with elevated serum glycolytic enzymes who did not have cancer, 5 patients with muscular dystrophy were given these materials over a period of several days to 1 week. There was no effect on the activity of any of the enzymes measured following the administration of either albumin or Amigen in amounts equivalent to those given to the patients with cancer. These studies will be presented in detail elsewhere.

Effect of human serum albumin and Amigen on serum enzymes in vitro.—To study the possibility that albumin and Amigen might decrease serum enzyme activity directly by inhibition, purified preparations of aldolase and LDH (obtained from Worthington Biochemical Corp.) were incubated for 1 hour with various concentrations of human serum albumin and of Amigen, and then assayed for activity. Within the limits of accuracy of the methods, no inhibitory effect of either albumin or Amigen could be demonstrated. For LDH, concentrations of human serum albumin at the level of, or higher than, those normally occurring or obtainable with infusion of albumin appeared to increase the measurable LDH level. Similarly, to serums containing high levels of enzyme activity were added variable amounts of either Amigen or albumin. Neither substance produced any decrease in the measurable enzyme activity in the incubated specimens.

Effect of tumor necrosis on serum enzyme activities.—After the feeding study, patient McGi received intravenous nitrogen mustard and exhibited a rapid decrease in size of lymph nodes. Daily serum enzyme determinations revealed a rapid fall in serum enzyme activity. Similar studies in 3 other patients demonstrated the same results, namely, that during the period of tumor shrinkage after nitrogen mustard the activity of the various glycolytic enzymes in serum fell. Similarly, in 2 patients who

received X-ray therapy for metastatic seminoma, both of whom showed marked clinical improvement, the activity of circulating enzymes fell to normal.

Discussion

There is an abundance of evidence in the literature that a growing tumor usurps the available protein from the diet, and, in the face of a diet inadequate to support tumor growth, may utilize body protein for its requirements (*14–17*).

In many but not all the patients studied, there was excellent correlation between the activity of glycolytic enzymes in the serum and the presence of significant and occasionally profound creatinuria. Protein feeding, either orally or parenterally in some but not all of the patients, corrected the serum enzyme abnormality and tended to decrease the creatinuria. In patients with muscular dystrophy who demonstrated both elevation of serum enzymes and creatinuria, similar protein feeding did not alter the serum enzyme activity or affect the creatinuria. *In vitro*, human serum albumin and Amigen had no effect on the activity of purified aldolase or LDH or on the measurable aldolase of LDH activity of human serums. It seems reasonable to conclude that: 1) Augmented protein intake, whether oral or parenteral, can, in many patients with cancer, correct the serum enzyme excess. 2) The reversal of these abnormal enzyme activities by protein feeding is specific for patients with cancer and not observed in muscular dystrophy. 3) This effect is not caused by a specific inhibition of the enzymes by the protein itself or by dilution of the enzymes in serum.

Sibley and Lehninger (*20–22*), Hill (*24*), Schade (*27*), and Bodansky (*23*) concluded that the high serum aldolase and phosphohexose isomerase seen in some cancer patients were related to necrosis of tumor. Certainly in those patients in whom protein feeding corrected the abnormality, it seems highly unlikely that necrosis was a major factor in the genesis of the abnormal enzyme activities. Second, those patients who received treatment with nitrogen mustard or X ray, in whom tumor dissolution was rapid, did not show a rise in serum enzymes but rather a fall. This fall could presumably be due to a direct effect of both X ray and nitrogen mustard on each of the enzymes. In the doses used, however, direct effects on enzymes have not been found.

With the above reservations, elevated serum glycolytic enzyme activities in patients with cancer may often be evidence of an internal negative nitrogen balance, and may lend support to the concept of a tumor as a nitrogen trap.

The late Dr. Robert Elman and his associates have reported on the effects of feeding terminal cancer patients (*28*). Most of their patients were able to take food through a gastric tube, and in most of them the administration of large amounts of protein calories resulted in increased well-being and a return of appetite. Similarly in this study, a few of our patients reported increased appetite after intravenous alimentation.

Of great interest in the present study was the apparent acceleration of tumor growth by the administration of large amounts of protein. This was seen in several of the patients, appearing immediately after dietary protein intake was increased. It was not seen when fat was given as a source of excess calories. Such a result might have been expected from the studies of Tannenbaum who showed that the level of dietary fat *per se* had no effect on the rate of growth of a transplanted tumor in mice (*29*).

However, impressive studies have demonstrated conclusively that, in animals, caloric and protein intake has a considerable effect on the growth rate of spontaneous and transplanted tumors (*8, 30, 31, 9, 15, 16, 10, 32, 33, 17*). Tannenbaum has shown that when mice bearing a mammary carcinoma were placed on a low calorie ration the growth rate of tumors was significantly diminished (*32*). White and Belkin (*17*), by placing mice on a diet almost free of protein, were able to demonstrate some inhibition of the rate of growth of mammary adenocarcinoma, but at the expense of severe weight reduction in the animal. Devik *et al.* have shown the same thing (*30, 31*).

The present study in humans appears to demonstrate enhancement of tumor growth by an artificially high protein intake. Most of the patients studied had poor appetites, had lost weight, had creatinuria and elevated serum glycolytic enzyme activities, all presumptive evidence of inadequate protein intake. Such patients might be considered to demonstrate the decreased rate of tumor growth seen in protein-calorie starved animals, and this decrease was reversed by addition of large amounts of protein to the dietary intake. The loss of appetite and decreased intake of protein and calories may be, in their net effect, a protection against rapid tumor growth.

Conclusion

Abnormal serum activity of aldolase, lactic dehydrogenase, and phosphohexose isomerase in many patients with cancer may be a result of and also an indicator of muscle wasting. As such, these abnormalities are evidence in humans of the extraordinary ability of tumor cells to obtain and retain protein for growth.

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Immunogenetic Studies on X-Irradiated Mice Treated with Homologous Hematopoietic Cells [1,2]

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Summary

An analysis was made of the immune reactions taking place in X-irradiated mice treated with homologous hematopoietic cells. The results of experiments with hybrid combinations of hosts and donors suggest that the grafted cells react immunologically against the isoantigens of the irradiated recipient. The production of anti-C3H agglutinins by the inoculated C57BL cells was demonstrated by the transfer of lymph nodes from the irradiated and treated C3H mice back to normal C57BL mice. The "cytotoxic" anti-host immune reaction responsible for the lethal "secondary disease" of homologously treated mice was demonstrated by the passive transfer of transplantation immunity produced by the isoantigens of the recipient. Bone marrow from irradiated C3H mice treated with C57BL cells conferred on other irradiated C3H mice a much shorter survival time than normal C57BL cells. It is concluded that the cells from the C57BL-treated C3H donors were immunologically activated by the primary C3H hosts and killed the secondary C3H hosts through an immunologically secondary response. The mortality of mice treated with mixed embryonic isologous and adult homologous cells indicates that even if, theoretically, a delayed antigraft response is feasible, the graft antihost response has a dominant role in the immunogenetic incompatibility. An attempt to overcome this incompatibility was made by injecting irradiated C3H mice with fetal C57BL liver cells. The increased survival time obtained in this experiment may suggest a method for the application of the principles of biological protection to animals of genetically heterogeneous populations.—**J. Nat. Cancer Inst. 21: 697–712, 1958.**

It is now well established that the lethal effect of total-body irradiation can be prevented by inoculating the irradiated animal, after exposure, with hematopoietic cells from a normal donor (*1*), and that complete

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protection can be obtained if the irradiated host and the inoculated cells are of the same genetic constitution (*2*). If, however, the inoculated cells differ genetically from the irradiated recipient, being from either a homologous or a heterologous donor, the lethal effect is postponed but is not prevented (*2*). The incompatibility demonstrated in this host-graft system has both theoretical and practical implications. The interest in the theoretical aspects stems from the fact that if this incompatibility is of an immunogenetic nature, there must exist here a rather unusual system of host-graft relationship, since total-body irradiation is known to suppress the capability of animals to respond immunologically to grafted tissues (*3*). From a practical point of view, the analysis of the mechanism of this incompatibility may provide methods for overcoming the immunogenetic barriers and subsequently these principles could be applied in the treatment of radiation injury and leukemia (*4*) in animals of heterogeneous populations.

This study was undertaken with the purpose of elucidating the mechanism of the immunogenetic incompatibility in mice protected by homologous cells. Our basic assumption was that in the treatment of irradiated hosts with foreign blood-forming cells, the grafted cells reconstitute the antibody-forming organs in the hosts. The somatic tissues of the recipient, being now foreign to the antibody-forming organs, will emit the antigenic stimulus and elicit in the antibody-forming cells an immune response of a cytotoxic nature that might be fatal to the host.

The concept of immune reactions of graft against host was the basis of the present investigation. During the course of our studies, Trentin (*5*, *6*) published his results indicating the existence of this postulated type of immune reactions in irradiated mice, and some of our experiments are based on principles similar to those of Trentin. In these experiments, however, the direct demonstration of the immunological nature of the genetic incompatibility is limited by the fact that this incompatibility results always in the death of the animal. Other experiments were therefore devised for a direct test, by means of adoptive immunity and other methods, of the immune reactions that take place in irradiated animals injected with homologous hematopoietic cells. The results served as a basis for further experiments designed to overcome the immunogenetic incompatibility in the protective treatment of total-body irradiation in mice.

Materials and Methods

The strain C3H and C57BL mice used in the present study were from the same source as in our previous studies (*7*, *8*). The mice, 3 to 4 months old, were irradiated in Lucite containers, exposed to 900 r of 220 kv., 15 ma., with a 1 mm. Al filter and an 0.5 mm. Cu filter; dose rate was 29 r per minute.

Spleen cells were prepared by breaking up the spleens of 8- to 9-day-old mice in Tyrode's solution with a Ten Broeck glass homogenizer. Bone-marrow cells were obtained from femurs of 6- to 8-week-old mice.

Tyrode suspensions of these cells were injected by tail vein 24 hours following irradiation.

Hemagglutinins were determined by the dextran-human serum method of Gorer and Mikulska (*9*).

Experiments

Treatment of C3H mice with isologous and homologous spleen cells.—C3H mice were inoculated 24 hours after irradiation with spleen cells of 8- to 10-day-old C3H or C57BL mice. Each recipient received an equivalent of 1 spleen. Although no complete protection was obtained in our experiment with isologous cells, 92 percent of the mice survived for more than 30 days, and more than half of these survived for more than 60 days. The maximal survival time in most experimental groups exceeded 120 days (table 1). On the other hand, of C3H mice treated with C57BL spleens, 83.8 percent survived less than 30 days. Since the irradiated, untreated controls showed a survival time of 8 to 9 days, it is concluded that treatment with homologous cells slightly increased the survival time of the irradiated C3H mice but did not confer any substantial protection.

TABLE 1.—*Effect of isologous and homologous spleen cells and of homologous embryonic liver cells on irradiated C3H mice*

Treatment	Number of experimental groups	Total No. of animals	Percent mortality at:			Maximal survival time in various experimental groups
			30 days	30–60 days	60–∞	
C3H spleen→C3H mice	13	75	8.0	37.3	54.7	>175, >178, >224, >260, >265
C57BL spleen→C3H mice	6	37	83.8	16.2	—	17, 21, 26, 28, 34, 37
Embryonic C57BL liver→C3H mice	5	33	12.1	66.7	21.2	49, 81, 89, >134, >248

Treatment of F_1 combinations.—In order to test the assumption that in treatment of C3H mice with C57BL cells the inoculated spleen cells react immunologically against C3H isoantigens of the host, hybrid combinations were irradiated and treated with cells of one of the parental strains. The assumption was that if the graft reacts against the host, then treating (C3H × C57BL)F_1 hybrids with C3H cells would confer no protection upon the recipient since the antigenic components of C57BL origin in the F_1 host are foreign to the C3H spleen, and may, therefore, elicit an immune response against the host cells. If, however, the incompatibility found in treating C3H mice with C57BL cells is due to the host's response, one would expect to obtain protection in this hybrid system. The results (table 2) show that no real protection was conferred on these animals. All of them died within 21 days following treatment. Similar experiments were made in which (C3H × C57BL)F_1 hybrids

were inoculated with spleen cells from C57BL, and (C3H × DBA)F_1 hybrids were inoculated with C3H cells. Animals of these 2 groups showed a longer survival time than those of the previous group, but none of them survived for more than 48 days. In fact, 10 to 12 of the (C3H × DBA)F_1 hybrids died within 30 days following treatment. It seems, therefore, that inoculation of hybrid mice with hematopoietic cells from one of the parental strains does not confer protection upon the irradiated recipients. The results of treatment of hybrid mice with parental spleen cells are, in principle, similar to those obtained by treating C3H mice with homologous spleen cells. This similarity is consistent with that obtained by bone-marrow treatment of irradiated animals (*10*).

Treatment of C3H mice with cells from (C3H × C57BL)F_1.—It can be assumed further that if the inoculated spleen cells are the responding elements in the immunogenetic incompatibility demonstrated in previous experiments, then treating C3H mice with cells from (C3H × C57BL)F_1

TABLE 2.—*Effect (a) of spleen cells from parental strains on irradiated F_1 mice, and (b) of F_1 spleen cells on irradiated C3H mice*

Treatment	Mortality at:			Maximal survival time (*days*)
	30 days	30–60 days	60–∝	
C3H→(C3H × C57BL)F_1	6/6	—	—	21
C57BL→(C3H × C57BL)F_1	5/10	5/10	—	48
C3H→(C3H × DBA)F_1	10/12	2/12	—	48
(C3H × C57BL)F_1→C3H (expt. 1)	4/15	5/15	6/15	>220 (4/15)
(C3H × C57BL)F_1→C3H (expt. 2)	2/9	2/9	5/9	>100 (4/9)

should result in a clear protective effect unlike the results of the reversed system. Such an experiment was carried out, and the results (table 2) show that 6 of 15 mice from experiment 1 survived for more than 60 days, 4 of which showed a survival time that has exceeded 220 days; 5 of 9 mice from experiment 2 survived for more than 60 days, 4 of which exceeded 100 days' survival time and are still alive. It is thus shown that treatment of C3H mice with spleen cells of F_1 origin can confer protection upon the irradiated animals, though the protective effect is not a general one.

Repopulation of C3H mice by F_1 hematopoietic cells.—The protection obtained by (C3H × C57BL)F_1 cells is attributed to the repopulation of the C3H hosts by the inoculated hematopoietic cells. No antihost response is possible in this case, since all the isoantigens of the host are included in the antibody-forming cells of the grafted tissue. However, a delayed host against graft response is theoretically feasible in this system, particularly in view of the results obtained in this study and in previous ones (*11*), showing that only part of the irradiated animals are effectively protected

by the F_1 cells. Such a response may take place even in the protected animals, but if it is accompanied by a regeneration of the host's hematopoietic tissue, it need not result in an interference with the protective effect accomplished by the F_1 cells. The final composition of blood cells of such animals will then be merely of the host's type. Moreover, even without the postulated delayed immune reaction, a regeneration of the host's hematopoietic tissue may cause a decrease or eventual elimination of the grafted F_1 cells. The final composition of blood cells in the protected animals would indicate whether such changes have taken place.

The analysis of the erythrocyte composition of C3H mice protected by (C3H × C57BL)F_1 was attempted by testing their agglutinogens with 1) antiserums against C3H-specific antigens and 2) antiserums against C57BL-specific antigens. Anti-C3H serum was, therefore, produced in C57BL mice, and anti-C57BL serum was produced in C3H mice. Each of these antiserums was tested for its specificity prior to analysis of the experimental animals. The red blood cells from 3 C3H mice treated with (C3H × C57BL)F_1 spleen cells were then tested 160 days after the protective inoculation. The results (table 3) show that both anti-C3H serum and anti-C57BL serum reacted strongly with the red blood cells of the experimental animals. The agglutination obtained with anti-C57BL serum clearly indicates the existence of (C3H × C57BL)F_1 erythrocytes, thus reflecting a functional repopulation by the hybrid-hematopoietic tissue. But since the anti-C3H serum might react both with F_1 and C3H cells, it is impossible to deduce from this experiment whether the reaction is due merely to F_1 cells or whether pure C3H blood cells have also appeared in the circulation. It should be noted, however, that although the anti-C57BL serum caused a very strong and complete agglutination, a successful separation of the agglutinated cells, in order to test for pure C3H cells in the remaining suspension, is impossible in this technique.

Demonstration of antibodies produced in graft antihost response.—The differences in the survival time between the two experimental F_1 groups, *i.e.*, between the F_1-donor group and the F_1-host group, definitely suggest that the graft responds immunologically against the isoantigens of the host, but these results are by no means proof of the existence of this immune reaction. It is evident that a direct demonstration of anti-C3H

TABLE 3.—*The agglutinogens of red blood cells of C3H mice protected by spleen cells from (C3H × C57BL)F_1**

Serum	Titer $>2^{10}$
C3H Anti-C57BL	+++†
C57BL Anti-C3H	+++
Normal C3H	0
Normal C57BL	0

*Tested 160 days after treatment.
†+++=Complete agglutination.

antibodies, elicited in irradiated C3H mice treated with C57BL spleen cells, would furnish much clearer evidence for the response of graft against host. It has been previously claimed, for a similar system (*12*), that such antibodies could not be found in the treated animal. We have assumed, however, that even if circulating antibodies against the host's tissues are formed in the treated animal in large quantities, one cannot expect them to be found in detectable amounts in the irradiated host due to their absorption by the host's somatic isoantigens that have elicited their production. Any attempt to demonstrate such antibodies must therefore be done in a system in which the C3H antigens will be eliminated. In order to achieve this, the following experiment was performed: C3H mice were irradiated and treated with C57BL spleen cells. Twelve days after the inoculation of the spleen cells, the animals were killed and the spleen and lymph nodes (2 inguinal and 4 axillar) were dissected, minced, and transferred by trocar intraperitoneally into normal C57BL hosts. Each C57BL mouse was inoculated with 18 lymph nodes and 3 spleens from 3 donors. It was postulated 1) that the antibody-forming cells from the C3H-irradiated host, if they are of C57BL origin, will not be destroyed immunologically in the C57BL hosts, and 2) that if these cells have been immunologically stimulated by the C3H isoantigens, and have been producing anti-C3H agglutinins, their passive transfer to C57BL hosts will enable the detection of the agglutinins in the circulation. The transferred cells will emit anti-C3H agglutinins that will remain free in the C57BL serum in the absence of their corresponding antigens. Control experiments were performed in which C57BL animals were implanted intraperitoneally with spleens and lymph nodes from normal C3H mice to assure that under similar conditions agglutinins are not produced by the C57BL host in response to the C3H tissue transferred in the experimental group. The animals of both groups were bled 4 and 5 days after the implantation and tested for agglutinins against C3H erythrocytes. The serum from the experimental group showed at the 4th day a titer of 1:16 and at the 5th day a titer of 1:124 of anti-C3H agglutinins. The serum from the control animals was completely negative, indicating that the implantation of C3H spleen tissue does not elicit any agglutinins in C57BL hosts within 5 days. It is thus shown that the lymph nodes and spleen cells from irradiated C3H mice treated with C57BL cells are immunologically stimulated by C3H antigens and produce anti-C3H agglutinins.

Cytotoxic graft-against-host response demonstrated by a "second-set effect."—It is generally accepted that the isohemagglutinins formed by homografts of normal or tumor tissues are not the antibodies responsible for the graft's destruction by the host's immune response. It is therefore evident that the antibodies demonstrated in the previous experiments are not the factors involved in the postulated immune destruction of the irradiated host by the grafted cells. It is generally known that no cytotoxic antibodies are found when a grafted tissue is rejected by the host's immune reaction. A destruction of a graft is attributed to an

immune response of the host if a second graft of an identical genetic origin is rejected by the same host within a shorter time. This increased resistance of the host, brought about the second graft, is known as the second-set effect (secondary response) and is the main criterion for the immune nature of the graft's rejection. It is, however, self-evident that a demonstration of a secondary response in the graft-against-host system is a rather complicated matter since the primary response causes the death of the animal. In order to overcome this limitation, an indirect demonstration of a secondary response was designed. It was postulated that if the irradiated C3H host is killed by the C57BL cells due to a "primary response," then if these cells from the treated animal are reinoculated, after exposure to the isoantigens of the first irradiated recipient, into another irradiated C3H animal, the survival time of the latter should be shorter than that of the C3H mice protected by normal C57BL cells. The following experiment was therefore carried out:

One group of C3H mice was exposed to 800 r (a dose lethal within 8–9 days) and inoculated after 24 hours with bone-marrow cells from normal C3H mice. A second group of C3H mice was similarly irradiated, but treated with bone-marrow cells from C57BL mice. Twelve days later the animals of these two groups were killed and their bone-marrow cells were used for the protective treatment of other irradiated C3H mice. A third group of C3H mice was then similarly irradiated and treated with normal C57BL cells. The treatment procedure for the three experimental groups is designated in the following plan:

	Donors	*Recipients*
(I)	(C3H⟶C3H)	⟶C3H
(II)	(C57BL⟶C3H)	⟶C3H
(III)	C57BL	⟶C3H

The results (table 4) show that C3H mice inoculated with bone marrow from irradiated C3H mice that have been previously treated with C3H cells (I) have a survival time exceeding 60 days. On the other hand, C3H mice inoculated with bone marrow from irradiated C3H mice that have been previously treated with C57BL cells (II) died within 13 days after bone-marrow inoculation. The C3H mice that have been similarly irradiated and protected by bone-marrow cells from normal C57BL mice (III) showed a mean survival time of 29.1 days, with a maximum of 34 days. The protection obtained by (C3H⟶C3H)⟶C3H treatment shows that bone-marrow cells from treated animals can confer protection upon irradiated animals. The much shorter survival time obtained by (C57BL⟶C3H)⟶C3H treatment (II), as compared to C57BL⟶C3H, can therefore be attributed to the fact that bone-marrow cells from (C57BL⟶C3H) were immunologically activated by C3H isoantigens of the primary host when drawn for inoculation of the secondary C3H mice. This shorter survival time of the secondary C3H recipients corresponds to the shorter survival time of a second skin graft (second-set effect) and may represent an immunologically secondary response.

TABLE 4.—*Effect of immunologically activated cells on irradiated C3H mice*

Treatment	Total No. of animals	Survival time of each animal (*days*)	Mean time of survival
(C3H⟶C3H)⟶C3H	7	44, >60 (6/7)	
(C57BL⟶C3H)⟶C3H	11	5, 5, 5, 5, 5, 6, 6, 6, 9, 9, 13	6.7
C57BL⟶C3H	9	22, 27, 27, 28, 28, 30, 33, 33, 34	29.1

The effect of mixed populations of isologous embryonic cells and homologous adult cells on survival of irradiated mice.—The previous experiments have clearly shown that inoculated cells can respond immunologically to the foreign isoantigens of the irradiated host. This does not, however, exclude the possibility of an immune response of the recipient against the isoantigens of the grafted cells. Furthermore, the lack of general protection in the C3H mice treated with F_1 cells certainly suggests the possibility of a delayed host-against-graft response. A further demonstration was therefore attempted of the *dominant* role that the antihost response plays in the immunogenetic incompatibility found by treatment with homologous cells. The experimental plan was as follows: One group of C3H mice was irradiated and treated with suspensions of mixed cell populations from C3H and C57BL donors, but whereas the C57BL cells were from an 8-day-old spleen, the C3H cells were from the liver of 17- to 19-day-old fetuses. Each mouse received an inoculum consisting of 7 parts C3H liver and 3 parts C57BL spleen. A second group of C3H mice were similarly irradiated and treated with mixed C3H and C57BL cells from 9-day-old spleens, the inoculum consisting of 7 parts C3H spleen and 3 parts C57BL spleen. A third group of C3H mice were irradiated and treated with embryonic C3H liver cells.

The assumption was that if embryonic C3H liver cells can confer protection on C3H mice, then the first experimental group of mice inoculated with mixed cell populations would provide a proper test for the relative potency of the two postulated immune reactions. The inoculated C3H cells are incapable of responding immunologically to the C57BL cells due to their embryonic state (*13*). But if the C3H host can react immunologically against the foreign graft, then the C57BL spleen cells would be destroyed and the remaining C3H embryonic cells would carry out the protective effect. On the other hand, if the antihost response is the dominant factor, although the C3H embryonic cells *can* by themselves protect an irradiated C3H mouse, the mere existence of C57BL cells will prevent this protection. Such a result will also eliminate the possibility that homologous cells do not protect because they fail to supply a hypothetical protective substance that might be metabolically incompatible with the irradiated foreign host (*14*). Had it been a matter of an "incompatible" substance, the existence of C3H embryonic cells would be sufficient to protect the C3H recipient in spite of the noneffectiveness of the C57BL factor.

The results (table 5) show that although embryonic C3H cells did protect the irradiated C3H mice, 15 of 23 irradiated C3H mice treated with embryonic C3H liver cells and C57BL spleen cells died within 60 days. The lack of protection in this group can be attributed only to the effect of the immune response of the graft against the host. Had the host reacted immunologically against the C57BL cells, the remaining C3H liver cells would have protected the C3H recipient.

TABLE 5.—*Effect of mixed cell populations of embryonic C3H liver and C57BL spleen on irradiated C3H mice*

Inoculated cells	Experimental groups	Mortality within 60 days	Percent mortality within 60 days
C3H embryonic liver + C57BL spleen	1 2	7/10 8/13	65. 2
C3H spleen + C57BL spleen	1 2	2/8 0/11	10. 5
C3H embryonic liver	1 2	2/8 2/10	22. 5

Three of 10 C3H mice (experimental group 1) treated with embryonic C3H cells and 9-day-old C57BL spleen cells and that survived for more than 60 days were tested for their red-blood-cell agglutinogens 38 days after treatment. The results (table 6) show that one of them contained a definite, although comparatively small, amount of C57BL erythrocytes; the second revealed traces of C57BL cells whereas the third did not show any signs of C57BL red blood cells. It is thus shown that in C3H mice treated with mixed populations of embryonic C3H and C57BL spleen cells, in contrast to C3H mice treated with mixed adult C3H and C57BL cells (*see* later), erythrocytes of C57BL origin can be found. The fact that, in the 3 C3H mice that have survived, the C57BL cells have either disappeared or remained in a small number may explain their survival.

In the second group of C3H mice treated with mixed populations of C3H and C57BL spleen cells (table 5), a definite protection was obtained;

TABLE 6.—*The agglutinogens of red blood cells of 3 of 10 C3H mice treated with mixed cell populations of embryonic C3H liver and C57BL spleen, that have survived for more than 60 days*

Serum	C57BL anti-C3H titer $>2^{10}$	C3H anti-C57BL titer $>2^{10}$	Normal C3H	Normal C57BL
Animal #1	+++*	+|†	−	−
Animal #2	+++	±	−	−
Animal #3	+++	−	−	−

*+++ = Complete agglutination.
†| = Half value of +.

17 of 19 mice survived for more than 60 days and are still alive. The explanation of the protective effect in this case is based on the mutual immune responses that might take place when mixed adult cells of isologous and homologous origin are inoculated into an irradiated host. This is described in the following experiment.

Protective effect of mixed spleen cells from isologous and homologous origin.—In order to study the protective effect of the mutual immune responses that are expected when irradiated mice are treated with mixed populations of adult cells, the following experiment was carried out: C3H mice were inoculated with suspensions of spleen cells from C3H and C57BL donors. Each mouse received the equivalent of 1.5 spleens, three quarters of a spleen from each donor. Controls were C3H mice treated with C3H spleen cells. The results (table 7) show a much greater protection in the experimental group demonstrated by a much longer survival time. The recovery of these mice was quicker than that of the controls, and seven eighths survived for more than 270 days.

TABLE 7.—*The protective effect of mixed cell populations of isologous and homologous spleens on irradiated C3H mice*

Treatment	Number of animals	Survival (*days*)
C3H → C3H	10	8, 34, 54, 69, 71, 73, 126, 224, 256, >270
C3H + C57BL → C3H	8	79, >270 (7/8)

A tentative explanation for the increased protective effect is based on the mutual immune responses that are expected when C3H and C57BL cells are inoculated. The C3H spleen cells will be stimulated by the C57BL isoantigens and will produce an anti-C57BL response. The C57BL spleen cells will similarly be stimulated by the C3H isoantigens from the whole somatic tissues of the host and will produce an anti-C3H response. But, whereas the target for the "cytotoxic antibodies" produced by the C57BL spleen is the whole organism of the host, the target for the antibodies produced by the C3H cells is merely the C57BL spleen cells. It is therefore conceivable that the C57BL cells will be destroyed by the C3H spleen cells before the anti-C3H response has produced its cytotoxic effect on the C3H host's tissues. The antigenic stimulation of the C3H spleen cells, in addition to its lethal effect on the homologous cells, will cause an augmented mitosis of the C3H spleen cells, thus leading to a quicker and more effective repopulation of the host's hematopoietic tissues. This increased rate of repopulation is probably responsible for the greater protection obtained by this treatment.

According to this viewpoint, C3H mice protected by mixed cell populations of C3H and C57BL donors should eventually contain only C3H blood cells. A test was therefore made of the composition of the red blood cells in these protected animals. C57BL anti-C3H and C3H anti-

C57BL serums were produced and used for agglutination tests of the red blood cells of the C3H mice, 38 and 200 days after their protective inoculation with the mixed suspension of C3H and C57BL cells. The erythrocytes reacted strongly with anti-C3H serum (table 8), but they did not show any reaction with anti-C57BL serum. In showing that the red blood cells of the protected C3H are completely of C3H origin, the serological results are consistent with the proposed interpretation of the immunological extinction of the C57BL cells.

TABLE 8.—*Agglutinogens of red blood cells of (a) C3H mice treated with mixed cell populations from C3H and C57BL donors, and (b) C3H mice treated with cells of embryonic C57BL liver*

Serum	Titer $>2^{10}$	
	Mixed cell population	Embryonic C57BL liver
C57BL Anti-C3H	+++*	–
C3H Anti-C57BL	–	+++
Normal C3H	–	–
Normal C57BL	–	–

* +++ = Complete agglutination.

Protective effect of homologous embryonic cells.—Having shown that the graft antihost response has a dominant role in the immunological incompatibility in irradiated mice, experiments were designed to overcome this incompatibility by treating irradiated animals with embryonic hematopoietic tissue from a homologous origin. The treatment with embryonic cells was based 1) on the fact that these cells are not capable of producing an immune response and 2) on the possibility that the embryonic cells, while maturing in the foreign host, may acquire immunological tolerance to the isoantigens of the irradiated animal. C3H mice were irradiated and inoculated with liver cells from 17- to 19-day-old C57BL fetuses. The results given in table 1 show that the mean survival time of mice thus treated is significantly extended in comparison to that obtained by adult spleen cells of C57BL donors. The main death period following the treatment with fetal liver cells occurs between 35 and 50 days, but the mice that escaped this period survived for more than 4 to 6 months.[3] Tests were carried out to determine the erythrocyte composition of C3H mice protected with C57BL embryonic liver, at 70 and 184 days following treatment. The antigens of the red blood cells of these animals were tested with anti-C3H and anti-C57BL serums. The results (table 8) show that no C3H agglutinogens could be detected in the C3H hosts that

[3] While completing this manuscript, we received the paper by Barnes *et al.* (*15*) on a similar approach to avoidance of "secondary disease" in radiation chimeras. They have obtained a clear, protective effect by inoculating irradiated CBA mice with newborn and embryonic spleen and embryonic liver cells from C57BL donors.

have survived after the inoculation of the C57BL liver, whereas the anti-C57BL serum caused a complete agglutination of the red blood cells. It is therefore concluded that in C3H mice treated with embryonic C57BL cells, which survived for 2 to 6 months, the C57BL cells have performed a complete repopulation of the C3H erythropoietic tissues and no regeneration has taken place in the host's red blood cells.

Discussion

The conception that the genetic incompatibility obtained by treatment of irradiated mice with homologous cells is due to an immune reaction stemmed from the demonstration that the protective agent in this treatment is a cellular one (*2*). The question of whether one is faced here with an ordinary host's immune response against the grafted cells (*12*) or with the rather unusual, postulated response of the grafted cells against the isoantigens of the recipient was thus the crucial point of dispute. The evidence indicating that the mortality caused by treatment with homologous hematopoietic cells is due to a graft antihost response was derived mainly from the findings that bone-marrow cells from hybrid donors can confer protection on mice of the parental strains (*11*), whereas the survival of hybrids treated with bone-marrow cells from their parental strains is similar to that of mice treated with homologous cells (*10*). Had the grafted cells constituted the only antibody-forming elements of the irradiated recipient, one would expect hybrid cells to protect mice of their parental strains to a similar degree as that of isologous cells. It has, however, been shown that this is not the case. Mice treated with cells from their hybrid donors showed a marked variation in behavior: Some were definitely protected whereas others were not. Furthermore, van Bekkum and Vos (*16*) found that the lethal effect of irradiating (C57BL $\times$ CBA)F_1 had been prevented by bone-marrow cells from either CBA or C57BL donors. This deviation in the results of the various hybrid experiments from those expected according to the graft antihost theory called for a more direct analysis of the immune responses produced by the "foreign" inoculated cells. This has been accomplished in the present study 1) by the detection of hemagglutinins produced by the homologous cells against the isoantigens of the host and 2) by demonstrating that a cytotoxic immune response is elicited in the inoculated cells against the isoantigens of the irradiated recipient.

Assuming that, in treating C3H mice with C57BL cells, hemagglutinins produced by the C57BL cells against the C3H antigens will be absorbed by the host's tissues, these antibodies were demonstrated by the transfer of the spleens from the treated C3H mice back to normal C57BL mice. Antibodies emitted by the transferred lymphoid cells were thus detected in the serum of the secondary C57BL hosts. It seems that the failure of Gengozian and Makinodan (*12*) to find antimouse agglutinins in irradiated mice that have been inoculated with rat bone marrow can be attributed to the absorption of the antibodies by the antigens of the host's somatic tissues. These authors, and Makinodan (*17*), claimed that no rat γ-

globulins could be demonstrated in mice treated with rat bone marrow. But this can also be interpreted by the assumption that most of the rat γ-globulins have been transformed into antimouse antibodies and are therefore absorbed by the host's tissues. Weyzen and Vos (*18*) have recently reported that rat serum proteins are produced in irradiated mice treated with rat bone-marrow cells.

Another approach to a direct demonstration of the immune response produced by the graft against the host was concerned with the cytotoxic reaction thought to be responsible for the "secondary disease" and death of mice treated with homologous cells. The transplantation immunity evoked by the host's antigens was demonstrated by the production of an immunological secondary response in irradiated mice. The injection of C3H mice with bone-marrow cells from other irradiated C3H mice that had previously been treated with C57BL cells resulted in a decreased survival time of the secondary recipient, indicating that the transferred cells were activated immunologically by the primary hosts. The survival time of the irradiated mice is thus shown to be determined by the level of transplantation immunity produced by the homologous bone-marrow cells against the isoantigens of the irradiated animal.

Experiments on the protection of irradiated mice with mixed populations of spleen cells from homologous and isologous donors have indicated the dominant activity of the antihost immune reaction. When irradiated C3H mice were inoculated with mixed spleen cells from C3H and C57BL mice, the latter were eliminated from the hematopoietic system, and the isologous cells remaining conferred a complete protection on the irradiated mice. The elimination of the homologous cells can be attributed only to the immune response produced by the grafted isologous cells, and not to an immune reaction produced by the host. This is deduced from the results of the second experiment with mixed cell populations in which the isologous cells were from embryonic C3H mice, whereas the homologous cells were from infant C57BL mice spleen. Had the host been the responding factor in the first experiment with mixed spleen cells, one would expect also in the second experiment a recovery effect, since after the immunological elimination of the adult C57BL cells, the embryonic C3H cells would be left, and these have been shown to protect irradiated C3H mice when injected by themselves. However, the results obtained in this experiment showed a lack of protection that can only be explained by the immune response of the grafted C57BL cells against the somatic tissues of the irradiated C3H mice. The isologous cells cannot respond immunologically against the homologous cells due to their embryonic state. Therefore no reaction against the C57BL cells can take place, and C57BL red blood cells have, accordingly, been found in these animals. On the other hand, the homologous cells can be activated by the C3H isoantigens and are therefore expected to produce an immune response that causes a lethal secondary disease. The result obtained in this experiment was, in fact, a lethal effect.

The immunological mechanism of the mortality occurring when irra-

diated animals are treated with isologous embryonic and homologous adult cells corresponds to the effect that Billingham and Brent (*19*) have obtained by injecting spleen cells from homologous donors into newborn mice, and to the lethal effect shown by Simonsen (*20*) when foreign spleen cells are inoculated into chick embryos. Here, also, the existence of the autologous embryonic cells of the host did not prevent the "immunological death" produced by the grafted foreign cells.

Prevention of the graft antihost response was attempted by treatment of irradiated C3H mice with homologous embryonic liver. The increased survival time obtained in these experiments and by Barnes *et al.* (*15*) can be attributed to the incapability of the embryonic antibody-forming cells to produce an immune response, and to the possibility that, while maturing in the foreign host, they acquire tolerance to the isoantigens of the host. An analysis of the blood-group composition of C3H mice treated with C57BL embryonic cells has shown that, 6 months after treatment, all the red blood cells of the C3H mice were of C57BL origin. Lindsley *et al.* (*21*) found that in rats treated with homologous bone marrow, 20 percent of the red blood cells were of the host type 147 days after treatment. On the other hand, Ford *et al.* (*22*, *23*) claimed that in treating mice with homologous cells, no lymphocytes of the host type could be found. The complete colonization of the protected animals obtained by embryonic foreign cells may have special significance in the treatment of leukemic animals (*4*). It is self-evident that, in the treatment of leukemia with normal hematopoietic cells following total-body irradiation, to prevent the regeneration of the host's blood cells is an essential factor.

The demonstration of a graft antihost response does not exclude the possibility of a delayed host-against-graft reaction. Dixon (*24*) has reported that if an antigen is injected into rabbits after a sublethal dose of total-body radiation, the recipients may acquire immunological tolerance to this antigen. Accordingly, one would expect, in animals treated with homologous embryonic or F_1 cells, that even if the host's antibody-forming cells do regenerate to some extent, they will acquire tolerance to the foreign isoantigens of the inoculated cells. However, Dixon (*24*) has pointed out that the time interval between irradiation and antigen injection may determine the immune reaction of the host. A too short time interval still results in antibody formation. It seems possible, therefore, that the observation of Congdon and Urso (*25*) that mice treated with rat bone marrow immediately after irradiation showed a shorter survival time than those injected 1 or 2 days after irradiation is also due to a stimulation of the host's immune reactivity. The partial recovery failure when mice are treated with F_1 cells, and the lack of complete recovery after treatment with embryonic homologous cells, suggests the possibility of a delayed host-against-graft response. The factors that may determine an effective development of immunological tolerance in the irradiated hosts to the foreign isoantigens of the injected cells are the subject of current experiments.

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Validity of Determining Circumcision Status by Questionnaire as Related to Epidemiological Studies of Cancer of the Cervix [1, 2]

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Summary

Comparison of patients' statements with examination findings concerning circumcision of a series of 213 male patients admitted to the Roswell Park Memorial Institute indicated disagreement between examination and statement in 34.4 percent of the patients. Persons whose statements agreed and disagreed with examination were similar in birthplace, languages read and spoken, socioeconomic category of occupation, and age. Analysis of the proportion of disagreeing patients examined by each physician indicated general similarity. Characteristics of patients who stated they were not circumcised, when the physician determined they were circumcised, were not significantly different from those who reported circumcision when the examiner recorded the reverse. However, the first-mentioned group was consistently different to a slight extent from the other in characteristics suggesting less familiarity with the English language. Also, there was a smaller proportion of professional workers in this group, and they were somewhat older. Further study regarding the relationship of circumcision to cervical cancer should be based on physical-examination findings with adequate definitions of degree of length of prepuce.—**J. Nat. Cancer Inst. 21: 713–720, 1958.**

A lower frequency of cancer of the cervix among Jewish women than among non-Jewish women has suggested the hypothesis that lack of circumcision of mates may be an etiological factor in this disease. To test this hypothesis, Wynder *et al.* (*1*) studied controls and several groups of patients with cervical cancer in several hospitals. They reported that the husbands of the cervical-cancer patients had a lower frequency of circumcision than did those of the controls. In their report there was a suggestion of difficulty in obtaining a valid history of the husband's circumcision status. They stated that 10 percent of the white non-

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Jewish patients and 20 to 30 percent of the Negro patients were unable to report on the circumcision status of their husbands. In an indirect check on the reports of circumcision status, they interviewed male patients and found that the frequency of circumcision among them was essentially the same as reported by the cancer patients and controls for their husbands.

As part of an epidemiological study of reported cases of cancer of the cervix in a community, we attempted to test the circumcision hypothesis by the interview approach. Our interviewers had the "clinical impression" that wives were quite uncertain concerning circumcision status of their husbands. We therefore considered it necessary to determine the validity of this type of information by a separate study. Obviously the best method of validation would be a comparison of the wife's answer with the results obtained by examination of her husband. Since there are practical difficulties in such a study, we thought that a first approach would be to compare results of examination with a person's statement concerning his own circumcision status. If a fair amount of disagreement were noted, one would expect somewhat more disagreement when wives' statements were compared with examination results. On the other hand, the absence of disagreement did not preclude the possibility of disagreement between wives' statements and examination results; a direct study of validity of wives' statements would then be necessary. This is a report of a study comparing persons' statements with examination findings regarding their own circumcision status.

Method of Study

Prior to their first visit to the Roswell Park Memorial Institute all patients are sent a questionnaire concerned with a variety of data for epidemiological studies; these include socioeconomic status, ethnic background, and circumcision. If the patient is a male, he is asked to place a check mark in a box to indicate whether he is or is not circumcised; if female, she is asked to report on her husband's circumcision status.

At their first visit, prior to being examined by a physician, patients are seen by an interviewer who reviews the answers on the questionnaire and then obtains information on additional subjects. After this, all patients routinely receive a complete physical examination.

Because of the admission procedure, it was possible to carry out the present study by requesting the examining physicians to pay particular attention to the condition of the foreskin and to record whether or not each patient was circumcised. No physician conducting these examinations had access to the questionnaire prior to examination, and he had no notion as to the patient's statement concerning his own circumcision status. Thus, it was possible to compare the statements of male patients on the questionnaire with the findings of examining physicians.

From January 10, 1958, when the study began, to March 11, when special recording of circumcision status ended, 237 male patients were referred to the Roswell Park Memorial Institute. Of these, the circumcision status of 14 was not recorded because of the absence of the recording

clerk on two occasions and because of confusion in setting up routine on the first day. Eight other individuals in the series were not included because they were young boys not routinely examined by the physicians participating in this study. Two additional persons were omitted because physicians were not certain about their circumcision status. Thus, the study population finally comprised 213 male patients.

Results

Comparisons of patients' statements with examination findings as to circumcision are presented in tables 1 and 2. Table 1 indicates that 8, or about 4 percent, of the 213 patients stated that they did not know their circumcision status. For 13, or about 6 percent, the data were incomplete for other reasons: a few patients were too ill on admission to complete the questionnaire and there were errors of omission by the patient and the interviewer. These 21 patients are eliminated from further analysis; the comparison of the examination results with patients' statements for the remaining 192 patients are presented in table 2. About 30 percent stated that they had been circumcised, but physicians reported that 44 percent had such evidence. Also, of the 192 patients, 34.4 percent disagreed with examination findings. About 25 percent of patients said they were not circumcised when examination indicated they were, and almost 10 percent stated they were circumcised when examination indicated the reverse. These relationsbips can be viewed in another way. Of the 84 patients who the physicians felt were circumcised, 47, or 56 percent, stated they

TABLE 1.—*Comparison of patients' statements with examination findings concerning circumcision status*

Examination finding	Patients' statements			No statements by patients	Total
	Circumcised	Not circumcised	Don't know		
Circumcised	37	47	3	9	96
Not circumcised	19	89	5	4	117
Total	56	136	8	13	213

TABLE 2.—*Comparison of patients' statements with examination findings concerning circumcision status*

Examination finding	Patients' statements				Total
	Circumcised		Not circumcised		
	Number	Percent	Number	Percent	
Circumcised	37	19.3	47	24.5	84
Not circumcised	19	9.9	89	46.3	108
Total	56	29.2	136	70.8	192

were not circumcised, and of the 108 patients determined as not being circumcised, 19, or 18 percent, stated they were circumcised.

The wide discrepancy between examination results and patients' statements led us to seek some possible explanation. One possibility was that the disagreement represented an error on the part of one or a few of the several physicians conducting the examinations. Comparison of the percent disagreements obtained by each of the examiners indicated that they had generally similar results (table 3).

TABLE 3.—*Disagreement between patients' statements and examination findings by physician regarding circumcision status*

Examining physician	Patients examined	Patients disagreeing	
		Number	Percent
A	60	19	31. 7
B	25	8	32. 0
C	29	9	31. 0
D	24	10	41. 7
E	46	18	39. 1
Others (include 6 physicians)	7	1	14. 3
Total	191*	65*	34. 0

*The name of the physician who examined 1 patient was not available.

Another possible explanation was that lack of familiarity with the English language led to a misunderstanding of the term circumcision. Information obtained on these patients permitted some analysis of this possibility by comparing those persons agreeing with the examiner with those who did not concerning birthplace, languages read, and languages used in their homes. The assumption in such comparisons was that persons who were foreign born, or who used a foreign language, might have less facility with English and hence would more likely err in interpreting the meaning of the term circumcision. These comparisons are presented in tables 4 to 6; no differences are noted.

TABLE 4.—*Birthplace of persons whose opinions agreed and disagreed with examination findings regarding circumcision status*

Patients' birthplace	Patients who agreed with examination findings		Patients who disagreed with examination findings	
	Number	Percent	Number	Percent
United States	102	81. 6	50	75. 7
Poland	1	0. 8	5	7. 6
Italy	8	6. 4	4	6. 1
Other	14	11. 2	7	10. 6
Total	125*	100. 0	66	100. 0

*On 1 additional agreeing patient no birthplace data were available.

TABLE 5.—*Languages read by patients who agreed and disagreed with examination findings regarding circumcision status*

Languages read	Patients who agreed with examination findings		Patients who disagreed with examination findings	
	Number	Percent	Number	Percent
English only	101	81. 5	52	80. 0
English + Polish	6	4. 8	7	10. 8
English + Italian	5	4. 0	2	3. 1
English + other	12	9. 7	4	6. 1
Total	124*	100. 0	65*	100. 0

*On 2 agreeing and 1 disagreeing patient no language data were available.

TABLE 6.—*Languages used at home by patients who agreed and disagreed with examination findings regarding circumcision status*

Languages used at home	Patients who agreed with examination findings		Patients who disagreed with examination findings	
	Number	Percent	Number	Percent
English only	78	63. 0	37	57. 0
English + German	5	4. 0	6	9. 2
English + Polish	10	8. 0	10	15. 4
English + Italian	13	10. 5	5	7. 6
English + other	18	14. 5	7	10. 8
Total	124*	100. 0	65*	100. 0

*On 2 agreeing and 1 disagreeing patient no language data were available.

It would be useful to examine agreeing and disagreeing patients regarding their education. Unfortunately, such data were not available. However, information on occupations was available, and since the socioeconomic category of occupations is highly correlated with educational status (*2*), an examination of agreement as related to occupation was undertaken. Table 7 indicates that agreeing and disagreeing groups had about the same proportion of professional workers, who usually have the most education, and persons in occupations requiring very little education.

Conceivably, patients who erred in interpreting the term circumcision could be elderly individuals who for one reason or another failed to understand the word. However, an examination of the age distributions of the groups that agreed and disagreed indicated no statistically significant differences. The mean age of persons agreeing with examination findings was 58.4 years as compared with 59.4 for those disagreeing. Standard errors for these means were 1.25 and 2.10, respectively.

It will be recalled that disagreeing patients fell into 2 categories: one group, the 25 percent who said they were not circumcised when physicians said they were, and the other group, the 10 percent who stated they were circumcised when examiners said they were not. It was our feeling that

TABLE 7.—*Occupational status of patients who agreed and disagreed with examination findings regarding circumcision*

Patients' occupations	Patients who agreed with examination findings		Patients who disagreed with examination findings	
	Number	Percent	Number	Percent
Professional workers	8	6.4	3	4.6
Managers	13	10.4	4	6.2
Clerks	6	4.8	3	4.6
Sales workers	5	4.0	3	4.6
Skilled workers	36	28.8	18	27.8
Operatives	22	17.6	15	23.1
Domestics, etc	11	8.8	7	10.7
Unskilled workers	17	13.6	5	7.7
Farmers	7	5.6	7	10.7
Total	125*	100.0	65*	100.0

*For 1 agreeing and 1 disagreeing patient no occupational data were available.

if a person did not understand the term circumcision, he was more likely to answer this question negatively; this would result in a higher percentage in the first group.

Comparison of these 2 groups, presented in table 8, indicates that the first group had a higher percentage of foreign born, of persons able to read a foreign language, and of persons using a foreign language at home. They also had a lower percentage of people in the professional and managerial class, and were somewhat older. Although none of these comparisons is statistically significant at probability levels of 0.05, each is in the direction suggesting that an "erroneous" negative answer may be related to lack of familiarity with English terminology.

TABLE 8.—*Selected characteristics of patients whose statements disagreed with examination findings*

Characteristic	Patient reported no circumcision: Physician reported circumcision ($n=47$)	Patient reported circumcision: Physician reported no circumcision ($n=19$)
Percent foreign born	25.5	21.0
Percent able to read a foreign language	23.9	10.5
Percent using foreign language in home	50.0	26.4
Percent professional workers and managers	6.7	21.1
Mean age and standard error	61.6 ± 2.3	55.3 ± 4.2

Discussion

The results of the present study indicate that in about 34 percent of patients there was disagreement between a patient's statement of his circumcision status and the determination of such status by examination. Persons disagreeing with examination findings did not differ appreciably

from those agreeing with respect to birthplace, languages spoken and read, occupation, and age. These variables appear to be involved in some way with capacity to understand English terminology, but it is still possible, of course, that persons unfamiliar with English and/or American forms and with the nature of circumcision could have produced some of the disagreement. This is suggested by the consistent, although statistically nonsignificant, differences between patients answering negatively when examination findings were positive and patients who gave a positive answer with negative examination findings. Nevertheless, these factors cannot account for all the disagreement found.

Another source of disagreement may lie in the examination procedure. It is possible that all the physicians could be equally mistaken in their findings. An uncircumcised male with a naturally short foreskin could be designated as circumcised. Again, the examiner observing a tight foreskin of moderate length in the case of a circumcised male, who had very little tissue removed, might designate him as uncircumcised. Interviews with examining physicians to ascertain their procedure in determining circumcision status did show some variation. In the majority of patients the glans was either fully exposed or covered, and a decision was readily made. In a certain proportion of patients the prepuce was long enough to allow doubt as to circumcision status. Such doubt was removed on the part of physician A by attempting to cover the glans with the foreskin; if he could not cover it he considered the patient to be circumcised. Physicians B and C routinely retracted the prepuce and examined the frenum. If the frenum had been sectioned, a judgment of circumcision was rendered. Physicians D and E, on the other hand, did not retract the foreskin but made a decision in the doubtful cases on the appearance of tightness of prepuce, on the presence of a flap of skin obviously left from an operation, and on evidence as to whether the frenum had been sectioned. Table 3 shows a statistically nonsignificant higher frequency of disagreement for physicians D and E than for the others. It is notable that despite variations in criteria, the percent disagreements for all the physicans did not differ significantly.

It is difficult to evaluate the relationship of the results of the investigation to epidemiological studies concerned with circumcision hypothesis in cancer of the cervix. In the study by Wynder *et al.* (*1*) circumcision status of the husband was determined by questioning the wife. As we indicated earlier, it is likely that a woman's statement concerning circumcision would probably be in greater error than that of her husband. One cannot dismiss the possibility that women might report circumcision status more accurately than the men themselves since they may obtain a more accurate conception of the nature of circumcision through a closer acquaintance with the operation during the neonatal period of their male children. However, it would be difficult to posit a greater degree of reliability on women's statements than on those of their husbands without more evidence on the subject.

Another factor to be considered is that errors of misclassification would

in general tend to diminish differences in comparisons between cancer and control groups, if they are random and unbiased (*3*). Thus, the result of a study showing no relationship between circumcision and cervical cancer might be a reflection of this kind of error, even if actually there was a positive relationship. On the other hand, our finding that the errors were not equally distributed between the patients who stated they were not circumcised, when examination indicated they were, and those who stated they were, when examination indicated the reverse, also influences evaluation of studies showing a positive relationship between circumcision and cervical cancer. In this instance, it is possible to obtain a spurious positive result. Even if several studies had such a positive result, assessment would be difficult since one would not know the direction and magnitude of the error in each of the studies. Of course, if the association is a very strong one, it is possible that the degree of error of misclassification may not be sufficient to cause concern. One cannot determine the type of situation that was present in the patient groups studied by Wynder *et al.* (*1*). However, it should be pointed out that the patients studied by Wynder *et al.* (*1*) were on the average about 10 to 15 years younger than those we studied, and perhaps in younger groups knowledge of the nature of circumcision might be more widespread.

Clearly the problem of determining circumcision status is complex and requires further study. It is also apparent that to test definitely the relationship of cervical cancer and circumcision, it is essential that information regarding circumcision status be obtained by examination of the husbands. In fact, from a biological viewpoint, it may be questionable whether one is really interested in the relationship of circumcision to cancer of the cervix. After all, circumcision is only an index of foreskin length. Since there are uncircumcised individuals with naturally short foreskins and individuals who were inadequately circumcised, it would seem necessary to measure the length of the foreskin or to develop well-defined categories of length. This is important since, as was indicated earlier, there were some variations in the criteria of circumcision used by our examining physicians. Inquiry of many other physicians indicates that these variations in criteria are widespread.

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Rate and Equilibrium Constants for the Reaction of Maleic Anhydride with the Methyl-1,2-Benzanthracenes [1, 2]

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Summary

The adducts of maleic anhydride and 1,2-benzanthracene, 9,10-dimethyl-1,2-benzanthracene and the twelve methyl-1,2-benzanthracenes were prepared. Second order rate constants for the reaction of these hydrocarbons with maleic anhydride in toluene at 100° C. were determined, as well as equilibrium constants at 180° in xylene. No correlation between these rate or equilibrium constants and recently determined carcinogenic activity of the hydrocarbons was observed.—**J. Nat. Cancer Inst. 21: 721–728, 1958.**

The search for some chemical or physical property of hydrocarbons that can be correlated with carcinogenic activity has long engaged the attention of research scientists (*1–7*). The difficulties inherent in the attempt to assign the degree of carcinogenic potency to any hydrocarbon are summarized in the "Introduction to the Survey on Carcinogenic Activity" by Hartwell (*8*). Part of the difficulties now have been overcome since von Haam (*9*) tested all the methyl-1,2-benzanthracenes and methylbenzo[*c*]phenanthrenes by two standardized methods of surface application and parenteral administration in pure-bred C3H mice. As a result of this work it seems desirable to list the hydrocarbons tested in three categories of carcinogenic activity, namely, *highly carcinogenic*, *moderately carcinogenic*, and *inactive*. A more precise differentiation at present seems unwarranted by the data reported (*9*).

In the 1,2-benzanthracene series (numbering shown) the 4-, 5-, 6-, and 10-methyl derivatives and the 9,10-dimethyl compound are *highly carcino-*

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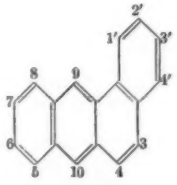
genic; the 9-methyl derivative is *moderately carcinogenic;* and the remainder, including the parent hydrocarbon, are *inactive.*



1,2-benzanthracene

The present work is a report on the relative rates of reaction of all the 1,2-benzanthracene derivatives with maleic anhydride in toluene at 100° C. and also on the equilibrium constants for the reaction in xylene at 180° C. in sealed tubes. This reaction was chosen because the site of reaction is the same for all derivatives, namely, the 9,10-position of the 1,2-benzanthracene nucleus. We wished to know whether there was any correlation between the rate or equilibrium constants and the carcinogenic activity of the compounds in question.

General Procedure for Rate Measurements

The rate constants were not obtained by the usual method of following the reaction at intervals. Rather, the extent of reaction was measured at only one suitable point. The rate constants were then calculated by the use of the integrated form of the second order rate equation,

$$k_2 = \frac{1}{t(a-b)} \ln \frac{b(a-x)}{a(b-x)}.$$

An 0.25 M solution of maleic anhydride was prepared by dissolving freshly sublimed maleic anhydride in dry toluene that had been distilled from and stored over metallic sodium. The concentration of maleic anhydride was determined by treating an aliquot with an equal volume of water and titrating the maleic acid formed with a standard solution of sodium hydroxide, with phenolphthalein as an indicator. The methyl-1,2-benzanthracene (0.303 gm.) was weighed into a 25 ml. volumetric flask; 10 ml. of the maleic anhydride solution introduced, and dry toluene added to the mark. A 10 ml. aliquot of this solution was pipetted into each of 2 reaction tubes [3] of about 100 ml. capacity. The molar ratio of maleic anhydride to hydrocarbon in this solution was about 2:1. The reaction tubes were immersed in an oil bath held at 100° C. ± 0.05° so that the oil level was 2 inches above the surface of the reaction mixture.

After a reaction time, *t*, of 3 hours,[4] the tubes were taken out and rapidly cooled in ice water; 10 ml. of water was added and rapid mechanical

[3] The tubes were fitted with a ground-in glass cap in the middle of which was sealed about a 2-foot section of 1 mm. capillary glass tubing which was bent in a helical coil and protected at the open end by a drying tube. Diffusion of solvent vapor from such tubes is negligible.

[4] A reaction time of one-quarter hour was used for 9-, 10-, and 9,10-dimethyl-1,2-benzanthracenes because the reactions were too nearly complete after longer reaction times.

stirring started. After stirring for a few minutes, the maleic acid in the mixture was titrated with 0.2 N sodium hydroxide. Alternatively, after the rapid cooling of the reaction tube, 10 ml. of 0.2 N sodium hydroxide was added to the reaction mixture and the mixture back-titrated with 0.1 N sulfuric acid. The latter method (back titration) gave more satisfactory results because with the former method (direct titration) the end point was frequently not sharp. Under the conditions used the adducts did not interfere in the titration.

Calculation of Rate Constants

Assuming that the thermal expansion of the reaction mixture was the same as that of the solvent, and using the density of toluene at 100° C., it was calculated that 10 ml. measured at 28° would expand to 10.90 ml. at 100°. However, at this temperature in the reaction tubes (*see* footnote 3), 0.25 ml. of toluene would have evaporated at 100°. The corrected volume of the sample at 100° would therefore be 10.65 ml. The corrected initial concentration of maleic anhydride was therefore about 0.0937 and about 0.0471 of hydrocarbon. The calculation of a typical second order rate constant is illustrated from results with 3-methyl-1,2-benzanthracene

$$k_2 = \frac{2.303}{t(a-b)} \log \frac{b(a-x)}{a(b-x)} = 0.46 \text{ c in l.}^{-1} \text{ hr.}^{-1}$$

where $t = 3$ hours; $a = 0.0937$ M maleic anhydride; $b = 0.0471$ M hydrocarbon; $x = 0.0055$ M adduct formed. The values taken for x are the average of 2 or more runs, which agreed with each other within 5 percent. The rate constants for all the hydrocarbons are listed in table 2.

Equilibrium Measurements

In these studies, the equilibrium points in xylene at 180° C. were approached by starting with mixtures of 1 mole of maleic anhydride and 0.5 mole of the hydrocarbon and also with mixtures of 0.5 mole of the adduct and 0.5 mole of maleic anhydride. The ratios of maleic anhydride to the hydrocarbon and to the adduct were chosen because of convenience and accuracy in titration.

The twelve methyl-1,2-benzanthracene-9,10-*endo*-(α,β-succinic anhydrides) and 9,10-dimethyl-1,2-benzanthracene-9,10-*endo*-(α,β-succinic anhydride) were prepared and purified by crystallization (table 1).

General Procedure for the Forward Reaction

All the pipettes and volumetric flasks were calibrated. For example, 3-methyl-1,2-benzanthracene (0.3034 gm.) was weighed into a 25 ml. volumetric flask, 10 ml. of a stock solution (0.25 M) of maleic anhydride in xylene was added, and the flask was filled to the mark with xylene. Thus, the concentrations of the hydrocarbon and the maleic anhydride

TABLE 1.—*Diels-Alder adducts of maleic anhydride and methyl-1,2-benzanthracenes*

Derivative	Analyses found*		Melting point range†‡
	C percent	H percent	
1′-Methyl	81. 4	4. 7	263–264°
2′-Methyl	80. 5	5. 1	217–225°
3′-Methyl	81. 6	4. 8	255–256°
4′-Methyl	81. 4	4. 7	253–255°
3-Methyl	81. 0	4. 9	258–259°
4-Methyl	81. 5	4. 9	214–215°
5-Methyl	81. 4	4. 8	247–250°
6-Methyl	81. 3	4. 6	246–247°
7-Methyl	80. 9	4. 6	243–244°
8-Methyl	81. 4	4. 9	236–246°
9-Methyl	81. 4	4. 9	244–246°
10-Methyl	80. 8	4. 5	274–277°
9,10-Dimethyl	81. 5	4. 9	238–239°

*Microanalyses by the Galbraith Laboratories, Knoxville, Tenn. Calculated for $C_{23}H_{16}O_3$: C, 81.2; H, 4.7. Calculated for $C_{24}H_{18}O_3$: C, 81.3; H, 5.1.

†Measured in sealed capillary pyrex tubes; the rate of heating was 1.5 to 2° per minute after the bath temperature had been raised rapidly to about 5° below the predetermined melting point range. All compounds decomposed somewhat at the temperatures involved.

‡The adducts were recrystallized mainly from ethyl acetate with varying amounts of alcohol added. The adduct from the 4-methyl compound was recrystallized from acetic acid. The yields were uniformly high but not exactly determined.

at 28° C. were found to be 0.0501 M and 0.0986 M, respectively. A 5 ml. aliquot was pipetted into each of three or four 20 ml. pyrex tubes. After sealing, the tubes were submerged in a fused salt bath held at 180 ± 1°. Every 24 hours, a tube was cooled rapidly and the maleic anhydride concentration was determined as follows: after the tube was opened, 10 ml. of 0.1 N sodium hydroxide solution was added and with the aid of water the mixture was quantitatively transferred to an Erlenmeyer flask. The alkaline mixture was then titrated with 0.1 N sulfuric acid. The average values obtained with the 14 hydrocarbons are shown in table 2. The fluctuations were rather large; in most cases the standard deviation was less than 4 percent except for the compounds 1′- and 6-methyl in which the standard deviation was about 9 percent. The reasons for deep colorations that were occasionally observed are not known. However, all results were included in obtaining the averages.

General Procedure for the Reverse Reaction

Eighty-five mg. of 3-methyl-1,2-benzanthracene-9,10-*endo*-(α,β-succinic anhydride) was weighed into each of three 20 ml. pyrex tubes. After adding a standard volume of 0.05 M maleic anhydride solution in xylene to each tube, they were sealed and placed into a fused salt bath held at 180 ± 1°. The adducts were practically insoluble in xylene at room temperature but the reaction mixture became homogeneous after a few minutes at 180°. It was assumed that the volume of the reaction mixture at room temperature (28°) was 5.0 ml., the sum of the pipette volume (4.97 ml.) plus the volume increment for added hydrocarbon.

A tube was removed after every 24-hour period and the amount of maleic anhydride present was determined as in the forward reaction. The fluctuations of the 0.1 N sulfuric acid titers were comparable to those obtained for the forward reactions.

Calculation of Equilibrium Constants

The equilibrium constant

$$K = \frac{[AD]}{[MA][MBA]}$$

where [AD] = concentration of adduct, [MA] = concentration of maleic anhydride, and [MBA] = concentration of hydrocarbon, was calculated from the titration data as follows: The forward case for 3-methyl-1,2-benzanthracene [MA] and [MBA] at 28° was 0.0986 and 0.0501 M, respectively. The concentration of maleic anhydride [MA] in the equilibrated mixture was obtained by the following equation:

$$[MA] = \frac{0.1003v_2 - 0.0996v_3}{2v_1}$$

where v_1 is the volume of the reaction mixture at 28°, v_2 is the volume of 0.1003 N sodium hydroxide added to the mixture after equilibration, and v_3 is the volume of 0.0996 N sulfuric acid required to back-titrate.

Substituting the values 4.97, 10.02, and 3.77 for v_1, v_2, and v_3, one obtains

$$[MA] = 0.0633 \text{ M}$$

and hence

$$[AD] = 0.0986 - 0.0633 = 0.0353 \text{ M}$$

and

$$[MBA] = 0.0501 - 0.0353 = 0.0148 \text{ M}.$$

However, corrections for the thermal expansion and the vaporization of the solvent were made in order to calculate the constant, K, at 180° C. It was assumed that the vapor pressure and the density of the mixture was the same as that of the solvent. Using the density as 0.715 [5] and the vapor pressure as 1800 mm.[6] at 180°, it was calculated that 5 ml. xylene at 28° would expand to 5.985 ml. at 180°. Assuming a free space of 15 ml., the volume of xylene vaporized would be 0.0013 ml. Since the latter is very small compared to the former, the volume at 180° was taken as 5.98 ml. Thus, the concentration at 180° was obtained by multiplying the values at 28° by 0.836 (5/5.98). The corrected values of [MA], [AD], and [MBA] thus calculated were 0.0529, 0.0295, and 0.0124 M, respectively.

[5] This value was obtained by extrapolating the 100-140° data in the International Critical Table for *p*- and *m*-xylene by a graphic method, assuming the curvature remains about constant.

[6] This was calculated by using $d_t = d_s + 10^{-3}\alpha(t-t_s) + 10^{-6}\beta(t-t_s)^2 + 10^{-9}\gamma(t-t_s)^3$, where $d_s = 0.8815$, $\alpha = -0.8515$, $\beta = -0.109$, $\gamma = -1.73$, and $t-t_s = 180°$ (*loc. cit.*) assuming this equation holds at 180°.

The K value obtained therefrom was 45 liter, mole^{-1}. In table 2 the K values are given for the 14 hydrocarbons. However, since the standard deviation of each K was about $\pm$ 30 percent on an average, these K values must be taken only as a qualitative aspect of the systems.

Similarly, K values for the reverse reactions were calculated from titration data and are given in table 2. Again these values deviate about $\pm$ 30 percent. Three tubes were evaluated for each K, both forward and reverse. The K values reported represent the numerical value of 3 determinations.

TABLE 2.—*Comparison of carcinogenic activity of methyl-1,2-benzanthracenes with rate constants and equilibrium constants for reactions with maleic anhydride*

Compound	Rate constant*	Equilibrium constant, K†			Carcinogenic activity‡
		Forward	Reverse	Average	
1′-Methyl	0. 78	70	70	70	−
2′-Methyl	0. 43	40	40	40	−
3′-Methyl	0. 47	40	60	50	−
4′-Methyl	0. 56	70	70	70	−
3-Methyl	0. 46	40	60	50	−
4-Methyl	0. 47	60	70	65	+++
5-Methyl	0. 54	70	60	65	+++
6-Methyl	0. 47	50	50	50	+++
7-Methyl	0. 47	50	40	45	−
8-Methyl	0. 56	80	90	85	−
9-Methyl	36. 80	120	150	135	+
10-Methyl	10. 40	240	—§	200§	+++
9,10-Dimethyl	100. 80	1500	—§	1500§	+++
Unsubstituted	0. 35‖	40		40¶	−

*Second order rate constants at 100° in toluene, k_2 in liter mole^{-1}, hour^{-1}.

†Equilibrium constants at 180° in xylene, K in liter mole^{-1}.

‡Carcinogenic activity determined by Dr. E. von Haam, The Ohio State University (*9*). The estimates of activity are ours: − = inactive or almost so; +++ = consistently carcinogenic by both skin painting and injection tests; + = moderately carcinogenic.

§Because of the large value, experimental difficulties made accuracy low. K's are markedly larger than in most cases studied.

‖Value in xylene previously determined by Dr. M. Cameron: other k_2 values in xylene were close enough to values obtained in toluene so that we believe this value is valid.

¶Determined only for forward approach.

Discussion of Results

Rate Data

If one uses the recently determined values (*9*) as a basis for carcinogenic activity, there is no correlation between rate of reaction of methyl-1, 2-benzanthracenes with maleic anhydride and carcinogenic activity. Thus, 4-methyl-, 5-methyl-, and 6-methyl-1,2-benzanthracenes have about the same low (relative) rates but are among the most potent carcinogens, while 10-methyl- and 9,10-dimethyl-1,2-benzanthracenes react at the highest rate and are also very potent carcinogens.

After calculating the electric charge at various positions in the 1,2-benzanthracene nucleus, Badger (*10*) attempted to correlate carcinogenic activity of methyl-1,2-benzanthracenes with the δq values obtained.

In that work, both 3-methyl- and 4-methyl-1,2-benzanthracene had approximately equal δq values and hence would be expected to have roughly equal carcinogenic activity. Since the carcinogenic activities to be reported by von Haam (*9*) and herein listed are at variance with other findings, we believe it best to defer a discussion of the relationship of carcinogenic activity to electron density as computed by theoretical means (*10*) until more data have been accumulated.

From a chemical standpoint, it is interesting that substitution at the meso positions of the anthracene nucleus increases markedly the rate of reaction whereas substitution by a methyl group at any other position leads to no marked acceleration. The enhanced reactivity of 9,10-dimethyl-1,2-benzanthracene with maleic anhydride is in line with previous observations (*11*, *12*).

The slightly higher rate for 1′-methyl-1,2-benzanthracene may be a result of the strain introduced by slight intramolecular overcrowding (*13*). In line with this observation is the fact that the 9-methyl compound is significantly more reactive than 10-methyl-1,2-benzanthracene.

Equilibrium Data

Of interest from a chemical standpoint are the high equilibrium constants for the meso-substituted derivatives as compared to all the other methyl-1,2-benzanthracenes.

As in the rate data, there is no correlation of equilibrium constants with carcinogenic activity nor is there any correlation between the equilibrium constants herein described and the equilibrium constants previously determined (*14*) for 2,4,7-trinitrofluorenone-methyl-1,2-benzanthracene complexes, 2,4,7-trinitrofluorenone-methylbenzo[*c*]phenanthrene complexes, and complexes of both series of hydrocarbons with silver ion (*7*). In discussing correlation of argentation constants with carcinogenic activity, Professor Lucas (*7*) did not use the carcinogenic activities determined by von Haam (*9*) since these were not available at that time. For this reason, the correlations noted by Lucas may now seem less convincing. However, before we discuss carcinogenic activity in comparison with chemical and physical properties for the methyl-1,2-benzanthracenes and methylbenzo[*c*]phenanthrenes, it appears desirable to await further accumulation of data on the carcinogenic activity of these hydrocarbons. We have quantities of all of them, which we will gladly supply to research workers in the field.

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Comparative Chromosomal Studies on Mammalian Cells in Culture. II. Mouse Sarcoma-Producing Cell Strains and Their Derivatives [1,2]

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Summary

Comparative cytological studies were made of mouse sarcoma-producing cell lines maintained in different media, their clonal derivatives, and the sarcomas produced by injection of these lines into mice of the original strain. These sublines were derived from one cell cultured from a cell strain that originated in the normal tissue of a mouse, but differences in morphology, metabolism, and in the frequency with which they produce sarcomas were demonstrated. Mitotic rate comparisons showed that all cell lines exhibited similar mitotic activities *in vitro*. In addition, mitotic abnormalities were observed in all lines. These lines may be classified as hyperdiploids, with most lines exhibiting characteristic modal chromosome numbers. Variable proportions of higher polyploid cells were found in all lines. Metacentric chromosomes are present in all lines, ranging on the average from 2 to 11 per cell in each line. Furthermore, certain characteristic marker chromosomes can be identified in individual lines. These can be detected in related strains and in the resulting sarcomas. These cell strains clearly have altered chromosome number and morphology as compared with normal mouse cells. Although these studies have established certain characteristic chromosomal differences between the high and low sarcoma-producing lines, there is no present evidence that these differences are directly responsible for their distinctive physiological properties.—**J. Nat. Cancer Inst. 21: 729–751, 1958.**

Transformations *in vitro* of normal mammalian-tissue cells to cells capable of giving rise to tumors have been reported by several workers (*1–4*). Sanford and coworkers (*5*) have further demonstrated that two sublines of a clone derived from a single mouse cell differ in their capacity to grow as sarcomas after implantation into mice of the inbred strain

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[3] Yale University.
[4] National Cancer Institute.
[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

(C3H) from which the cells originated. One line designated "high" produced sarcomas in 97 percent of the mice injected; the other, designated "low," in 1 percent. Later, cells of the high line showed a slight decline in sarcoma-producing capacity. Both cell lines induced immunity in strain C3H mice, but cells of the high line increased in numbers more rapidly when implanted and appeared to produce an established tumor before resistance developed in the host. It is recognized that such established tumors can continue growth after immunity develops. On the other hand, cells of the low line grew so slowly on transplantation that the cells apparently succumbed to the immunity induced in the host. Cell proliferation rates *in vitro*, based on nuclear counts, were approximately the same in both lines. The two lines of cells have also been shown to differ in cell morphology, rates of anaerobic and aerobic glycolysis (*6*), and glucose utilization (*7*).

Subsequently, 3 clones from the low line and 1 clone from the high line were derived by means of single-cell isolations. In order to grow the single cells, it was found necessary to use chemically defined medium NCTC 109 (*8*) supplemented with 10 percent horse serum rather than the medium of horse serum, chick-embryo extract, and balanced saline in which the cells had been cultured previously. In contrast to the parental lines, cells of the clones were found to grow progressively in a higher percentage of the mice injected. This increased transplantability of the clones could have resulted from the different culture medium used, and when the parent lines were placed on this medium, a similar change in transplantability gradually took place. However, a significant difference between the two lines in metabolic properties and in their rate of growth into tumors was maintained in the new medium.

Since chromosomal changes might have occurred in these cells that could be responsible for, or associated with, their differences in neoplastic properties and transplantability, studies of the chromosomes of these cells were undertaken. Information regarding chromosome number and morphology might provide additional criteria for establishing relationships as well as differences among these cell lines, and might help to elucidate the mechanisms responsible for their origin and behavior.

Materials and Methods

The origin and early history of the high and low lines (now designated NCTC strains 1742 and 2049, respectively) have been described (*5*). The cells were maintained in a medium consisting of 40 percent Earle's balanced saline, 40 percent horse serum, and 20 percent chick-embryo extract (1:1.25) (*9*). NCTC strains 2672 and 2673 were derived from high and low lines, respectively, and were grown in chemically defined medium NCTC 109 (*8*) supplemented with 10 percent horse serum. NCTC clone 2472 originated from a cell of the high line after 6 years *in vitro*, whereas NCTC clones 2445, 2470, and 2555 originated from single cells of the low line isolated also after 6 years. All the clones were maintained in medium NCTC 109 supplemented with 10 percent horse serum.

The cells of 3 sarcomas were examined. The first sarcoma was produced by injecting high-line cells (after 7 years *in vitro*) into the thigh muscle of a strain C3H mouse. In 15 days a palpable tumor developed at the site of injection and 8 weeks later was removed and dissected for study. The second sarcoma was produced by injecting low-line cells (after 7 years *in vitro*) into a nonirradiated mouse. A palpable tumor developed in 6 weeks and was dissected 5½ weeks later. The third sarcoma was produced by injecting low-line cells into a mouse that had received 425 r of X radiation, and the tumor was first palpable after 18 days. Three and one-half weeks later it was removed and dissected.

In order to eliminate any cellular changes that might occur during shipment and cultivation under different conditions, all experiments and cytological preparations were made at the National Cancer Institute. Prepared slides and fixed materials were brought to New Haven for further analysis.

In the usual way with equal inoculum size and prescribed media, cells of different lines were seeded into T-9 flasks on the same day. Mitotic indices were estimated by counting metaphase configurations from duplicate samples harvested on 6 consecutive days and then on the 8th day after inoculation. Cells from each flask were collected and stained with aceto-orcein. More than 1,000 cells were enumerated in each sample for this purpose.

For chromosome studies, the cells in flasks were pretreated with colchicine (final concentration, 10^{-4} gm./liter of medium) for 1 to 2 hours before fixation. The cells were then removed from the walls of flasks by moderate shaking, collected, and incubated at 37° C. for half an hour with a hypotonic saline (1 part of Earle's saline with 9 parts of Earle's saline lacking NaCl). The treated cells were either fixed in 1:3 acetic acid-alcohol or fix-stained, and then squashed in aceto-orcein on slides as temporary preparations. The concentration of aceto-orcein was approximately 1 percent in 45 percent acetic acid and contained about 2 percent by volume of 1 N HCl to minimize cell breakage during squashing. Acetic acid-alcohol fixed cells were stained with acetocarmine.

Sarcomas freshly dissected from animals were immediately placed in Earle's saline at room temperature and cut into pieces about 1 to 2 $mm.^3$, and pretreated with hypotonic saline in the same manner described. The tumors were either squashed in aceto-orcein according to the method of Tjio and Levan (*10*) or fixed in acetic acid-alcohol, Feulgen-stained, and squashed in 45 percent acetic acid.

Chromosome counts were made from single, intact cells at metaphase or prometaphase in which the chromosomes were widely spread. A sketch was made of each cell counted, in order to check the chromosome number and morphology as accurately as possible. However, counting errors of the order of 1 or 2 chromosomes per cell may occur. The error may be slightly greater in high polyploid cells. Photomicrographs were taken with a Leitz Ortholux-Aristophot unit.

Results

Mitotic Activities

Mitotic rate comparisons, in terms of percentage of metaphase configurations at one time, have shown that all cell lines show the highest percentage of cells in division on the 1st day after seeding. The activity declines rapidly on the following day but increases somewhat after medium renewal in the culture. With the exception of NCTC clone 2472, which exhibited the highest rate on the 1st day after seeding, comparisons between other lines failed to show clear differences in mitotic activities under uniform conditions with respect to inoculum size, but with different culture media. This observation is in agreement with the more accurate quantitative results based on nuclear counts (*7*) which indicated that the high and low lines appeared to have the same proliferation rates *in vitro*.

In addition, mitotic anomalies, such as spindle multipolarity, endoreduplication, multinucleation, giant-cell formation, and nuclear fragmentation, have been frequently observed in all cell lines.

Chromosome Number and Morphology

The chromosome number and morphology found in these cell lines are presented in table 1. Both the high and low lines and most of their derivatives are found to be heteroploids. A modal chromosome number, with a scattered distribution of different chromosome numbers around the peak, seems to exist in most lines. The modal chromosome number of the high line (NCTC 1742) appeared to be 56, while that of the low line (NCTC 2049) was 49. In both, when the same line is maintained in a different medium, in this instance a serum-supplemented, chemically defined medium, there are shifts of the chromosome-number distribution pattern. The high line in chemically defined medium (NCTC 2672) seems to have a wider spread of chromosome numbers with no apparent mode in the present sampling size. There are increased numbers of higher than tetraploid cells in NCTC 2672 derived from the high line, but not in NCTC 2673 from the low line.

On the other hand, the clonal derivatives seem to possess a distinct modal chromosome number and pattern of distribution. The clone (NCTC 2472) from the high line shows a slightly lower mode but with increased proportion of higher polyploid cells than does the parental line. The 3 clonal derivatives from the low line also show characteristic distinctions from the parental line. NCTC clone 2555 exhibits an even more striking difference with a high modal chromosome number—69.

Variable proportions of higher polyploid cells are found in all cell lines. When the percentages of cells with chromosome numbers higher than tetraploid ($4n = 80$) are tabulated, the high line and its derivatives seem to show a higher proportion than do the low line and derivatives. However, in the sample examined NCTC clone 2470 contains a very high (37%) proportion of such cells.

Chromosomes with median or submedian centromeres are present in all lines, but the number seems to vary from line to line and from cell to cell within each line. The range and modal numbers of metacentrics per cell in each line are also indicated in table 1. In addition, there are other chromosomes with a characteristic morphology which serve as marker chromosomes. For example, figures 1 and 2 show two cells at metaphase from the high line with metacentric and telocentric chromosomes and two minute, nearly telocentric chromosomes. These two minutes (m1 = 0.7μ, m2 = 1.1μ in length) are present in the majority of cells in high line and its derivatives (figs. 3 and 5). Figure 6 is a cell from NCTC 2672 (high line in serum-supplemented, chemically defined medium) showing 65 diplochromosomes as a result of endoreduplication. Here two m1 and two m2 chromosomes can be identified.

In the high line (NCTC 1742), higher polyploid cells with chromosome numbers over 150 were often encountered. In these higher polyploid cells, excessive chromosome structural changes and fragmentations were observed. A ring chromosome has been repeatedly found in many such cells (fig. 4).

In the low-line cells, there exist on the average a higher number of metacentrics. Characteristically, one very small, nearly telocentric chromosome m3 (about 0.5μ in length) is present in the low line (figs. 7 and 8) and its derivatives (figs. 9 and 10). Additionally, it is possible to identify some characteristic metacentrics in certain lines, such as, for example, a markedly large chromosome (about 8μ in length) with a median centromere in the clonal line NCTC 2445. NCTC clone 2555 from the low line shows a very high average number (11) of metacentric chromosomes per cell. One of these metacentrics is a very long one (about 10μ) with a submedian centromere (fig. 10). Both these very long metacentrics can be traced in the low-line cells. It should be noted that metacentric chromosomes and minute chromosomes are not found in normal mouse cells, which have 40 telocentric chromosomes with the average length from 1.9 to 4.9μ (*11*).

The more limited chromosome studies of the sarcomas produced by injections of high- and low-line cells are indicated in table 2. All 3 sarcomas studied seem to show an equally wide range of chromosome numbers. With the present sample size, it is difficult to establish the modal chromosome number. Nevertheless, sarcoma #2 produced by injecting low-line cells seems to exhibit a distinct modal chromosome number different from that of the low line. There are also present a number of metacentric chromosomes in the sarcomas, with 2 to 6 in sarcoma #1, 1 to 4 in #2, and 3 to 6 in #3. Interestingly enough, the characteristic marker minute chromosomes are also found in cells of the resulting sarcomas (figs. 11 and 12). The existence of variable heteroploid chromosome numbers, metacentric chromosomes, and especially the characteristic marker minute chromosomes clearly indicate the origin and cell lineage of the resulting sarcomas.

TABLE 1.—*Chromosome number and morphology in mouse sarcoma-producing cell lines and their derivatives*

Strain No. (NCTC)	Description	Culture medium*	Chromosome number																												
			41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
1742	High line	I										2	1	2	1	6	4	10	9	4	3			2	1						
2672	High line	II			1				1	3	1	3	5	3	4	4	1	1			1		1		1			3			1
2472	Clone from high line	II				1			1		1		3	3	4	2	6	5	3	1	2		1								
2049	Low line	I		1		1	1	2	6	10	12	7	1	1	1																
2673	Low line	II	1	1	1	1		1	1	3	3	8	3	7	3	11	5	5	6	3	1			1							
2445	Clone from low line	II						1	1	2	7	2	3	10	13	5	1	4	1	1										1	
2555	Clone from low line	II																						1	1			1	2	5	14
2470	Clone from low line	II						2		1	2	1		6		2	1		1								1				

Strain No. (NCTC)	Chromosome number																							Percent of cells higher than tetraploid	Range of metacentrics per cell	Modal No. of metacentrics per cell	Avg. No. of small chromosomes (0.5–1.5μ in length) per cell	No. of cells analyzed
	70	71	72	73	74	75	76	77	78	79	80	81–90	91–100	101–110	111–120	121–130	131–140	141–150	151–160	161–170	171–180	181–190	>190					
1742								1					1	1	1			1		3				13.2	1–6	3	2	51
2672		3				2					3	18	7	2	1									40.0	1–9	4	2	70
2472													2	5	1									19.0	0–2	2	2	42
2049												2												4.4	5–10	6	1	45
2673												1		1		1								4.4	1–4	1	1	68
2445								1						4								1		8.6	1–5	2	1	58
2555	5	5	3	8	2		1		1														1	2.0	8–14	11	1	50
2470												1	6	3										37.0	2–4	2	1	27

*Culture medium I:

Earle's saline	40%
horse serum	40%
chick-embryo extract (1:1.25)	20%

Culture medium II:

chemically defined medium NCTC 109	90%
horse serum	10%

TABLE 2.—*Chromosome number and morphology in mouse sarcomas produced by injections of cultured mouse cells*

Sarcoma designation	Produced by injections of cells from	Chromosome number																						
		<40	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1	High line (NCTC 1742)			1	2				1			2	2			1	3	1	2	1		3	1
2	Low line (NCTC 2049)	1		1		2	1			1	1	2	4	5	5	9	1	2	2			1	
3	Low line (NCTC 2049)		1	1		1	1								1								

Sarcoma designation	Chromosome number														Range of meta-centrics per cell	Modal No. of meta-centrics per cell	Number of cells examined
	61	62	63	64	65	66	67	68	69	70	71 75	76 80	81 85	86 90			
1	3				1		1		1	1	2		1		2–6	3	30
2		1										1		1	1–4	2	41
3													1		3–6	2	6

Discussion

Transformations *in vitro* of normal mammalian-tissue cells with respect to their morphology and tumor-producing capacity have been reported in the literature. In recent years, cytological examinations of many established and rapidly proliferating mammalian-cell strains have revealed the existence of extensive heteroploidy, with very few or no euploids (*12–16*). The finding of heteroploidy in strains of cells cultured from normal tissues implies profound changes during the prolonged period of cultivation, because primary cultures of normal tissues always show a very high percentage of diploid cells (*17–20*).

Recent cytological studies of both human (*21*) and mouse (*22*) primary cultures have clearly demonstrated cellular abnormalities and heteroploid formation during the early *in vitro* cultivation. Cytological aberrations as well as fluctuations in nuclear DNA content have been shown to occur in primary cultures of monkey kidney cells (*23*).

In the present studies, the cell strains under investigation have been demonstrated to possess altered chromosome number and morphology as compared with normal mouse cells. Although the cytological conditions of the original cultures from which these cell lines were derived are not known, the findings of heteroploidy in all the present cell lines originated from normal C3H mouse tissue presumably represent additional instances of cellular changes during cultivation. The cells of both high and low lines were found (*7*) to immunize C3H mice. Such altered genetic constitutions of the cell lines may explain the origin of the immunological incompatibility between the two cell lines and the mice of strain C3H from which they originated.

On the other hand, although these studies have established certain characteristic chromosomal differences between the high and low lines and their derivatives, there is no present evidence that these differences are directly responsible for the distinctive physiological properties of the various lines. Studies on the karyology of neoplasms both in rodents and in man immediately indicate that most neoplasms contain chromosomal anomalies. Moore and coworkers (*24, 25*) have further associated tumor-forming capacities with heteroploid conditions in various human cell strains. However, it is clearly not possible to conclude that all cytologically transformed cell strains are neoplasms. The results of the present study indicate that deviating chromosome numbers and other anomalies exist in both high and low lines. There is, in fact, a certain degree of overlapping in the chromosome-number frequency distributions of the two lines.

The additional question of whether the higher frequencies of higher polyploid cells (accompanied by further chromosomal structural changes) are associated with sarcoma-producing capacity cannot yet be answered. Squash preparations of a sarcoma produced by injecting high-line cells failed to show such high polyploid cells. A number of studies (*26–30*) have demonstrated that an increase in chromosome ploidy may widen the

host specificity of transplantable tumors in the mouse. In the present studies, the sarcoma-producing capacity of the cell lines is found to be strain-specific. Although the high line and its derivatives did show a higher proportion of cells with chromosome numbers over 80, one of the clones from the low line (NCTC 2470) seems to consist of a large proportion of higher polyploid cells. However, this same clone has recently been shown to exhibit a shortened tumor latent period and higher anaerobic glycolysis rate as compared with its parental low line.

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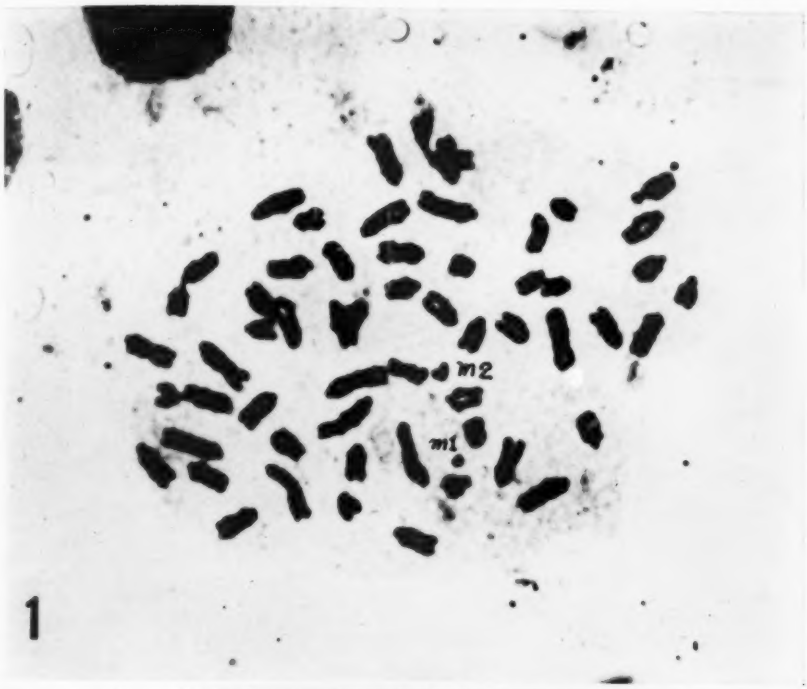
PLATES

PLATE 66

FIGURE 1.—A cell from the high line (NCTC 1742) with 57 chromosomes including 5 metacentrics and 2 minute (m1, m2) chromosomes. M1 measures about 0.7μ and m2, about 1.1μ in length. × 1,950

FIGURE 2.—Another cell from the high line showing 53 chromosomes including 5 metacentrics. Only one minute chromosome (m1) is identified in this cell. × 1,950



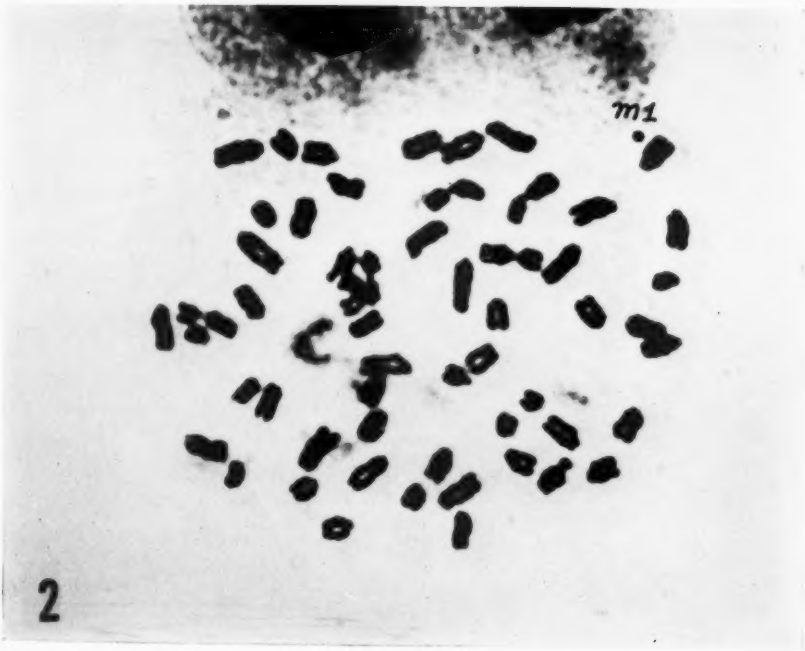


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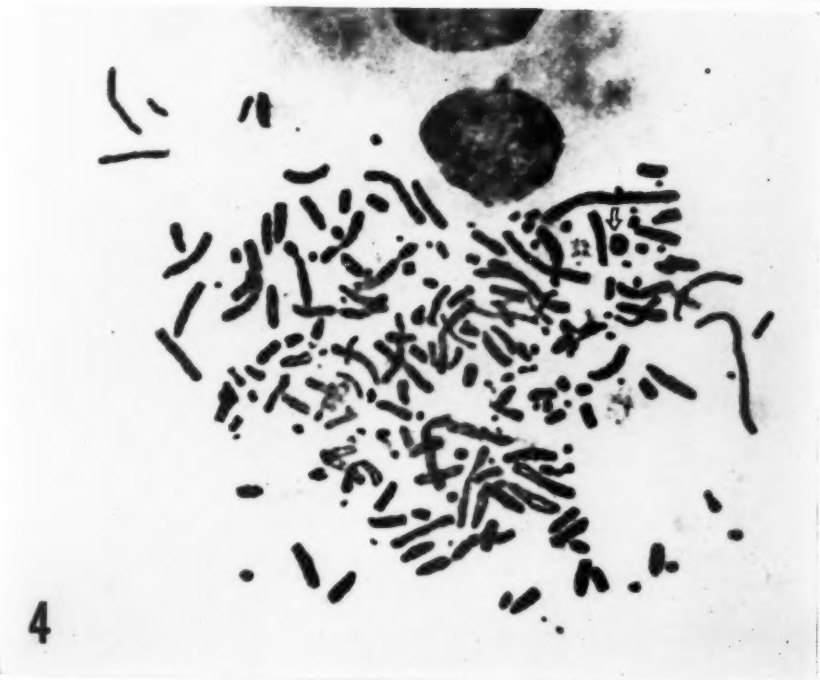
PLATE 67

FIGURE 3.—A cell from NCTC 2672, which is the high line maintained in serum-supplemented, chemically defined medium. Fifty chromosomes are present including 2 minutes and 6 metacentrics. × 1,950

FIGURE 4.—A high polyploid cell from the high line (NCTC 1742) with excessive chromosome fragmentation and structural rearrangements. *Arrow* indicates a ring chromosome. × 1,225

3

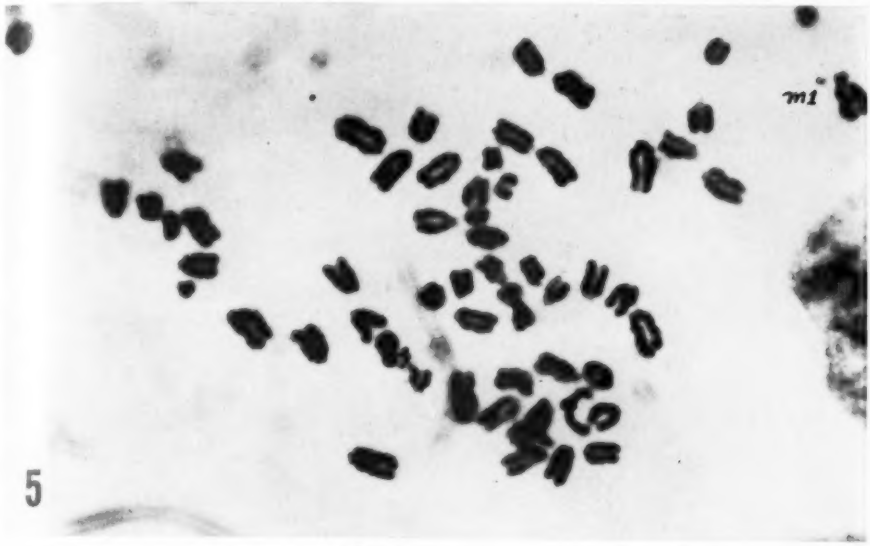


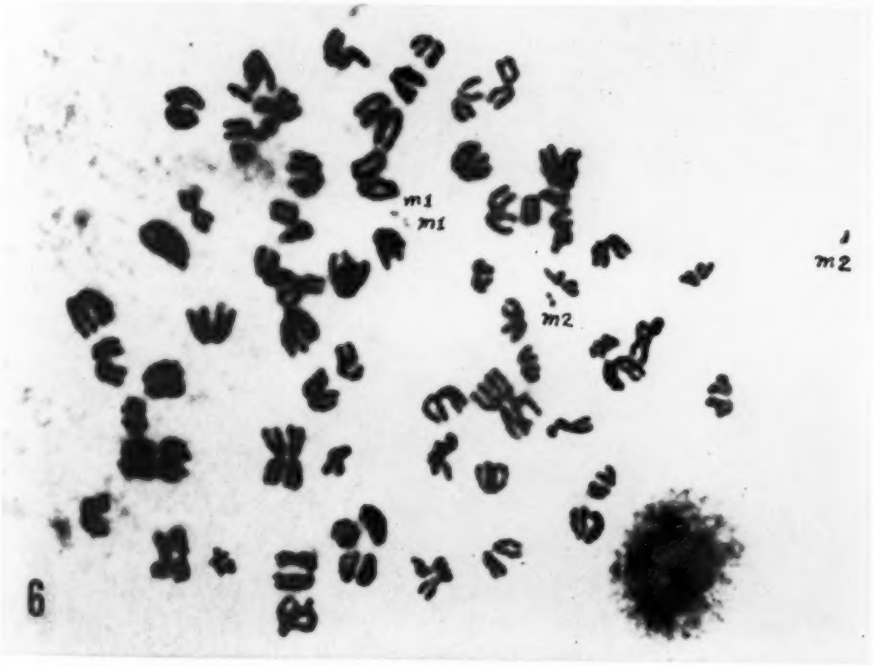
4

PLATE 68

FIGURE 5.—A cell from NCTC clone 2472 derived from the high line. It has 59 chromosomes including one metacentric and one minute (m1). × 1,950

FIGURE 6.—A cell from NCTC 2672 with 65 diplochromosomes. Two m1's and two m2's can be identified. × 1,650



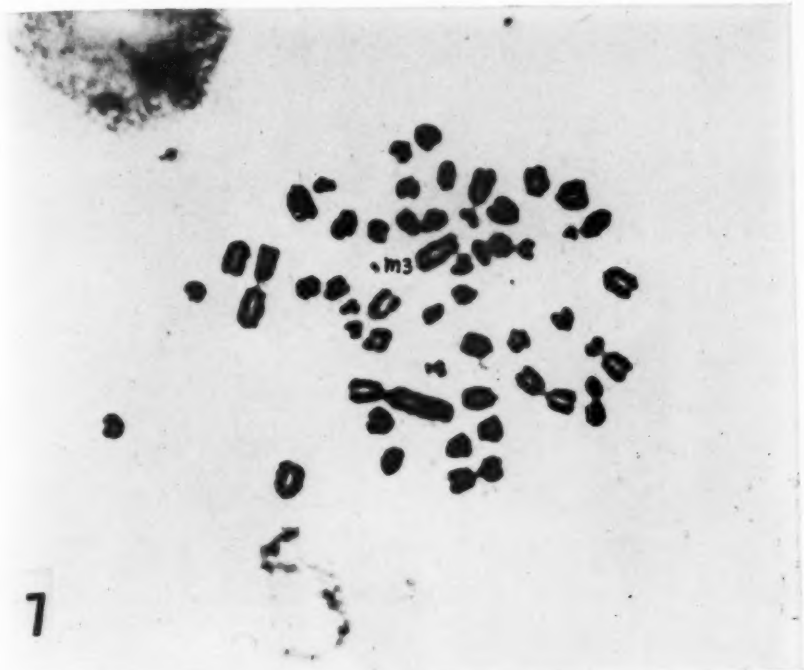


475402—58——10

PLATE 69

FIGURE 7.—A cell from the low line (NCTC 2049) showing 49 chromosomes. It contains 8 metacentrics and one minute (m3). M3 measures about 0.5μ in length and is smaller than both m1 and m2. × 1,950

FIGURE 8.—Another cell from low line showing the minute (m3) and a ring chromosome (*arrow*). × 1,950


m3
7

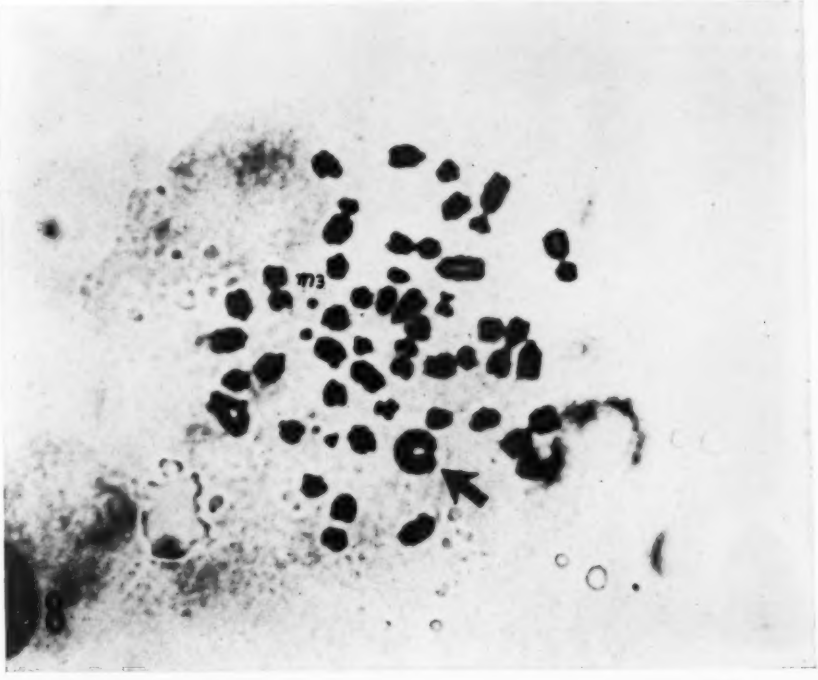
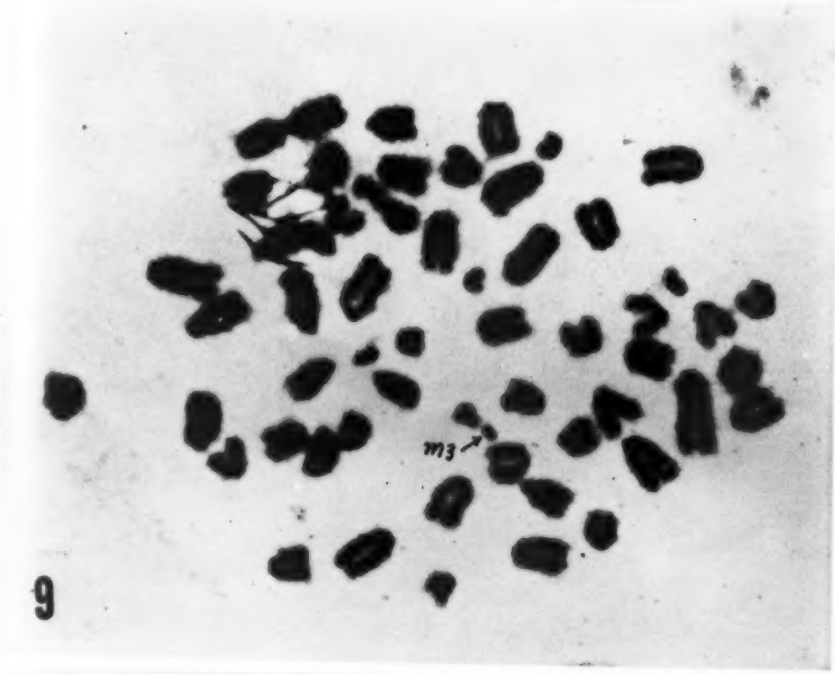

m3
8

PLATE 70

FIGURE 9.—A cell from NCTC 2673 which is the low line maintained in serum-supplemented, chemically defined medium. Fifty-seven chromosomes including one metacentric and one minute (m3) are present. × 1,950

FIGURE 10.—A cell from NCTC clone 2555 derived from the low line, showing 69 chromosomes with 10 metacentrics. *Arrow* indicates the longest (10μ) metacentric and points to the position of the submedian centromere. × 1,950


m3
9

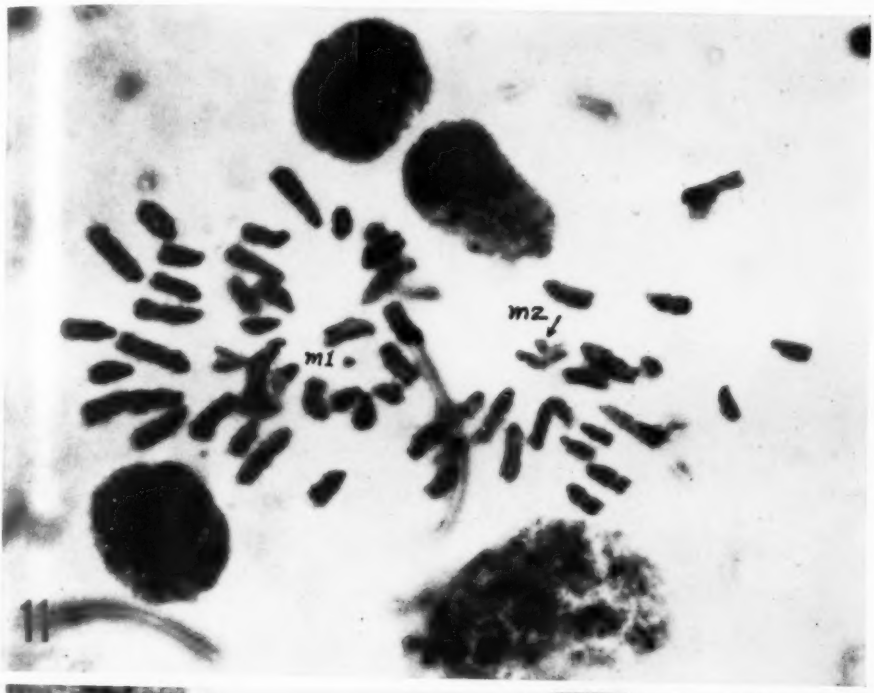
10

PLATE 71

Feulgen-squash preparations of hypotonic-saline pretreated sarcoma cells.

FIGURE 11.—A cell from sarcoma #1 produced by injections of high-line (NCTC 1742) cells. Fifty-nine chromosomes with two metacentric and two minutes (m1, m2) are shown. × 1,950

FIGURE 12.—A cell from sarcoma #2 produced by injection of low-line (NCTC 2049) cells. Fifty chromosomes including one metacentric and one minute (m3) are present. × 1,950


m1
m2
11

m3
12

Progressions of a Reticulum-Cell Sarcoma of the Mouse *In Vivo* and *In Vitro* [1]

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Summary

A histiocytic neoplasm, induced by methylcholanthrene in a DBA/2 mouse and adapted to ascites form, has been observed in serial mouse transfer and in continuous culture for 2 years. *In vivo*, the neoplasm showed a minor amount of morphologic progression toward a less well-differentiated histiocytic form. *In vitro*, morphologic progression was marked by the appearance of cells with single or multiple giant nuclei. Ascites tumors resulting from inoculation of cultures into mice 11 months after explantation showed greater morphologic change than the tumors carried continuously in mice during the same period. Changes consisted in increased pleomorphism, the occurrence of numerous giant cells, and the loss of distinctive histiocytic features. Ascites tumors derived from cultures at 11 months showed a nearly pure hypotetraploid population, compared with a predominantly hyperdiploid population in the serially mouse-passed line at comparable time. Mitoses in cells in culture after 24 months showed chromosome numbers predominantly in the near-diploid range. Metacentric chromosomes were found both in long-term cultures and in ascites tumors derived from cultures, but not in the serially mouse-passed line. In continuous mouse passage the ascites tumor showed at the 15th and 69th transfers nearly equal virulence. After 8 and 11 months in continuous culture, the ascites tumors derived from these cultures showed a marked decrease of virulence. After 11 months or more in culture, inoculums of 10^6 culture cells into mice rarely resulted in tumor production. Mechanisms of origin of the observed changes of latent period, virulence, and tumor-producing ability are discussed.—**J. Nat. Cancer Inst. 21: 753–781, 1958.**

Continuous cell culture of lymphomas and leukemias of laboratory mice has been little used in the investigation of these neoplasms. One reason may be that experimentalists recognize that the study of neoplastic cell strains in culture is not equivalent to the study of neoplastic disease in

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a living animal. Probably, however, the chief reason is that few workers have been successful in isolating continuous culture strains of murine lymphomas and leukemias. Valuable information might be expected from studies of isogenic strains of such neoplasms carried simultaneously *in vitro* and *in vivo*. The observations to be reported here are based on this type of approach.

Bichel (*1*) in 1938 and in the following years (*2*, *3*) first reported the continuous cultivation of a mouse leukemia. This was the Krebs leukemia, which had been classified as granulocytic at the time it was put into tissue culture, but was later designated stem cell in type (*4*). In later papers, Bichel reported cultivation of a highly undifferentiated plasma-cell leukemia (*5*), and of a chloroleukemia (*4*) obtained from Furth. In all these studies the leukemic cells could be propagated only by repeatedly adding normal mesenchymal cells to the culture vessels. These represented some of the earliest examples of *in vitro* "feeder systems."

De Bruyn in 1949 (*6*), using methods similar to Bichel's, was able to grow cells of the T 86157 (MB) lymphosarcoma continuously. She later succeeded (*7*, *8*) in isolating a pure cell strain, MB-III, without the feeder system, but this strain over a period of 2 to 3 months lost its ability to produce neoplasms when transferred back to mice of the appropriate genetic constitution. Since then, de Bruyn (*9*, *10*) has reported isolation of still another variant, MB-VI, which not only grows independently in suspension cultures but also retains the ability to grow and produce lymphosarcomas in mice. She has also reported (*9*) continuous cultivation of a myeloid leukemia without the support of normal mesenchymal cells.

More recently, Fischer (*11*) has reported the cultivation of L5178, a lymphoid leukemia, in suspension-type cultures and without the use of a feeder cell. He has emphasized the importance of citrovorum factor and peptones in the nutrition of this cell strain (*12*).

In 2 preliminary communications (*13*, *14*), the continuous cultivation of 2 reticular neoplasms of DBA/2 mice was described. The present paper describes alterations observed in the first of these, a reticulum-cell sarcoma, carried continuously for 2 years in tissue culture and through 61 transfers in the mouse during this same period.

Materials and Methods

Neoplasm

Classification.—P329 is an example of the histiocytic or Type A reticulum-cell sarcoma of the mouse according to Dunn's classification (*15*). It is analogous to the clasmatocytic type of reticulum-cell sarcoma in Gall and Mallory's classification (*16*) of human lymphomas.

History.—The original neoplasm arose in a female DBA/2 mouse at the age of 8 months, after 5 skin paintings with a 0.2 percent solution of methylcholanthrene in ether during the first 2 weeks of life. In the first 2 mouse passages, transfer was accomplished by subcutaneous injection of liver brei. In the third and fourth passages it was converted to the ascites form

by transferring cell suspensions intraperitoneally. The subsequent pathways through which the neoplasm was directed *in vivo* and *in vitro* are depicted in text-figure 1.

Gross morphology.—In the original host at autopsy the liver and spleen were enlarged and studded with pale-yellow nodules that projected above the external surfaces of these organs. The mesenteric lymph node was slightly enlarged. The thymus gland and peripheral lymph nodes showed no significant enlargement. The abdominal cavity contained less than 1.0 ml. of bloody fluid.

In the earlier passages of P329, intraperitoneal inoculation resulted in the accumulation of 3 to 5 ml. of ascitic fluid, containing approximately 10^8 cells per ml. The liver generally contained multiple small, yellowish-white nodular tumor deposits, but hepatic enlargement was only moderate and the parenchyma was not extensively replaced. Neoplastic deposits were also regularly found in the spleen, and in the mesenteric, caudal, lumbar, renal, and mediastinal lymph nodes, but there was little or no involvement of superficial nodes, such as the cervical, axillary, brachial, and inguinal nodes. Focal nodular replacements of the bone marrow of the sternum and vertebral bodies were generally found. The gastric and genital omenta were regularly heavily infiltrated with neoplastic tissue, there were subserosal infiltrates throughout the abdominal cavity, and there was invariably a solid mass of neoplasm, measuring 1.0 cm. or more in diameter, at the base of the mesentery. The uterus and ovaries were usually enveloped in a neoplastic sheath. Other organs were generally not involved, grossly or microscopically. Terminally, neoplastic cells were demonstrable in smears of the peripheral blood, but their number was not sufficient to cause elevation of the white-cell count. Characteristic tumor ascites could be initiated by intraperitoneal inoculation of peripheral blood from animals carrying the neoplasm.

Microscopic morphology.—In the original host, and in the earlier passages, the type-cell of the tumor was remarkably similar to the normal histiocyte of the mouse. In the spleen, liver, and lymph nodes, the tumor cells generally had an epithelioid appearance, with abundant eosinophilic cytoplasm and centrally placed nuclei (figs. 1 and 2). The nuclei were relatively small, often lobulated or reniform, with rather finely divided and evenly distributed chromatin and 1 or 2 small nucleoli. In the liver and occasionally at other sites, foci were found in which the cells were predominantly spindle-shaped and in the aggregate gave a picture mimicking fibrosarcoma. Multinucleated forms, while not rare, did not form a conspicuous part of the picture. In the larger tumor masses in the mesentery and omenta, yellow-orange deposits of crystalline material, described by Dunn (*15*) as characteristic of these tumors, were often found.

Wright's-stained smears of P329 ascites showed a uniform population of histiocytoid forms with voluminous wavy-edged cytoplasm, often indistinct at the periphery (fig. 4). The cytoplasm generally was only moderately basophilic, with a gray overcast, and usually contained numerous clear vacuoles. Nuclei showed a fine reticular chromatin pattern inter-

rupted by several granular chromocenters. One to three relatively small, distinct, blue-staining nucleoli were present as a rule.

Cultures

The medium used throughout the work reported here was made up of the following materials: pooled adult human serum, 40.0 percent; Hanks' balanced salt solution, 53.0 percent; glutamine, 0.015 percent; chick-embryo extract, 1.5 percent; beef-embryo extract, 0.5 percent; chicken plasma, 1.5 percent. Penicillin G was added in quantity sufficient to give a final concentration of 100 units per ml., and phenol red to give a final concentration of 0.001 percent. When the above components were mixed, the embryo extracts and chicken plasma were added last; the final mixture was rapidly shaken and immediately pipetted into tubes or bottles in 10.0 ml. or 100.0 ml. volumes. A clot then formed, which, on contraction, cleared the medium of microscopic particulate matter. The clot was discarded from individual containers after reopening, just prior to use. The medium was stored in an electric freezer at −20° C.

Three types of glass vessels were used. During the first 8 months after isolation, cultures were carried entirely in roller tubes closed with white rubber stoppers. Thereafter, cultures were also grown on glass surfaces of flat-bottomed tubes (Leighton) and 2-ounce medicine bottles, also closed with white rubber stoppers.

Explantation Procedure

A tumor nodule from the liver was cut into explants measuring approximately 1.0 mm. in diameter. These were placed in contact with the edges of a strip of gelatin sponge (Gelfoam) [5] about 2.5 cm. above the bottom of each roller tube. The gelatin-sponge strips measured 2 to 3 mm. in width, 1 to 2 mm. in thickness, and 2.0 cm. in length subsequent to thorough soaking in medium. When 6 to 8 explants and sponge had been properly arranged on the tube wall, the aggregation was clotted against the glass, with a mixture of 1 part chicken plasma to 2 parts of 20 percent chick-embryo extract. Excess clotting mixture was removed as soon as clotting began to occur, leaving only a thin membranous clot covering the circumference of the tube and the explants. Plasma clot was not used in subsequent handling of cultures, and use of gelatin sponge was limited to the initial tubes. Roller tubes were rotated at 12 r.p.h. Incubator temparature during the first 8 months of culture was 35.5° C., and 37.5° C. thereafter.

Observations and Results

Cells in Culture

Zero to 3 months.—The group of cultures from which the continuous line of P329 eventually emerged consisted of 6 gelatin-sponge, roller-tube

[5] Upjohn Company, Kalamazoo, Mich.

preparations as described. In all tubes there was a very active migration and apparent proliferation of cells from the explants during the first week of culture. These actively migrating cells, as seen in living cultures under low magnification (100×), were amoeboid and resembled normal macrophages. However, the migration of these macrophagelike cells was much heavier than the macrophage migration from any other normal or neoplastic tissue that we have observed in culture, including other types of leukemia. Within a period of 3 days to a week after explantation, the entire lower halves of the roller tubes were heavily coated with a relatively uniform layer of these amoeboid cells. Also during this period the plasma clot was almost completely removed.

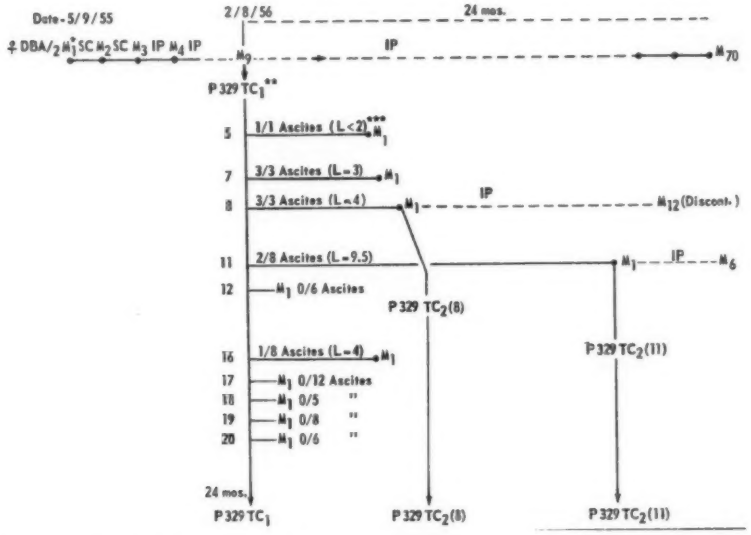
Progressive enlargement of the migratory histiocytoid cells was noted during the first month of culture. When they first appeared at the edges of the explants, these cells were relatively small, approximately 14 to 18 microns in diameter, and displayed rather short, hairlike cytoplasmic processes. They then became larger and more irregular in shape until by the end of the month the majority exceeded 35 microns in diameter. Large pseudopodia extending in different directions and generally having blunted tips were noted in most cells. Whereas the nuclei had not been clearly distinguishable at the beginning of culturing, most cells after the first month showed a large, clear, circular area, nearly central in position. This central clear zone was bounded by a ring of granular material having a faint yellow-to-brownish coloration. The granular material generally did not extend into the long, filamentous, cytoplasmic processes. Staining procedures carried out after the culture line had become established showed that the yellow-brown granules were fat droplets, and that the central clear areas represented the nucleus and a juxtanuclear or centrospheral area to be described in more detail. In living cultures, it was difficult to distinguish the nucleus from the juxtanuclear clear zone. Occasionally one could see 2 to 5 nuclei in a single cell. This observation was confirmed by stained preparations of later cultures.

After 3 to 6 weeks in culture, the cell populations in all tubes began to decline. During this phase, many cells became very large, up to 100 microns or more in diameter, and developed numerous long, sometimes branched, cytoplasmic processes (fig. 7). Such cells appeared to represent the aging members of the population. They could often be recognized in varying stages of disintegration while still adherent to the glass. By the 6th week, the gelatin sponges had become infiltrated by cells so heavily filled with yellowish-brown cytoplasmic "granules" (fat droplets) that visibility within the sponges was greatly reduced or lost.

Three to 5 months.—At 3 months, only a few scattered amoeboid cells persisted on the glass, except in a narrow zone immediately around the gelatin sponges where cells were more numerous. At this time also, the sponges had largely lost their compartmental structure and had collapsed into jellylike films. The majority of surviving cells was located between these films and the glass tube walls. All cells were now granular and amoeboid. At this time, a batch of medium was used in which the serum

component contained excessive fat. The cells not covered by gelatin sponge took up fat from the medium and swelled tremendously, apparently being incapable of refusing the fat. Ultimately these cells aggregated in spherical groups and then disintegrated, as though bursting from sheer oversize. Cells underlying the residual fragment of sponge were apparently protected from the fatty medium, and during the subsequent months these cells gradually emerged around the margins of the sponge.

Five months to 1 year.—After 5 months of culture, only 2 of the original 6 tubes contained living cells, limited to an estimated 100 cells or less in each tube. By means of small but frequent feedings and careful maintenance of the *p*H in the region of 7.2 to 7.6, the surviving cells were encouraged to increase until, 7 months after explantation, each tube had a coating of cells extending completely around its lower half. The first subcultures were made at this time, simply by scraping and washing the tube walls with a pipette. The serial subcultures derived from 1 of these original 2 tubes have been continued as cell strain P329 TC1 (text-fig. 1).



TEXT-FIGURE 1.—Schematic representation of tissue culture and mouse passage of P329. * "M" indicates mouse passage; ** "TC" indicates tissue-culture passage; *** "L" indicates latent period in months for appearance of gross ascites. Mouse passages are indicated on horizontal lines. Continuous tissue-culture passages are indicated on vertical lines.

The second, third, and fourth serial subcultures of P329 were accomplished at intervals of 2, 1, and 1 months after the first, and, since the tenth serial transfer, it has been possible to subculture at weekly intervals. However, since we have been more interested in trying to retain the original characteristics of the cells than in obtaining maximal proliferation, serial

transfers have been made at longer intervals. After 24 months of cultivation, only 24 serial subcultures were completed, an average of about 1 transfer per month.

At the sixth serial transfer, it was found that standard trypsinization methods were applicable to removal of P329 cells from glass surfaces, with a final concentration of trypsin varying between 0.035 and 0.1 percent depending on the density of cell populations. However, cells in trypsinized suspensions had some tendency to aggregate in small clumps, reducing the accuracy and precision of cell counts.

One year to 2 years.—As already stated, in the early weeks after isolation, the histiocytic forms enlarged greatly and resembled macrophages in having many long cytoplasmic processes and conspicuous centrospheral areas. Multinucleated forms were numerous. As the early subcultures became heavily populated, it was noted that cells in the densest areas were in most instances round and much smaller in diameter than cells in sparsely populated areas. With successive shortening of the period required for subculturing, the proportion of small, round cells to large, amoeboid cells became progressively greater (fig. 8). It was apparent, however, that the small, round cells were capable of transforming to the larger type cells when seeded from dilute suspensions. Under this condition, nearly all round forms converted to sprawling, histiocytoid forms. Stained coverslip cultures have shown that the great majority of mitoses occurs in the small, round cells, although occasionally they have been seen in spindle forms.

Morphology of Fixed Cultures

With certain exceptions, the major nuclear and cytoplasmic characteristics of P329 cells in culture have remained quite similar to those already described for the neoplastic cells in the ascites of the early mouse transfer generations. In wet-fixed coverslip cultures (figs. 9, 10, and 16), the nuclei show a decided uniformity of size in spite of wide variations in the area covered by cytoplasm. The nuclei are most often round, but are sometimes elongated and somewhat lobulated or indented. Nuclear membranes in hematoxylin-stained preparations are distinct, and 1 to 3 nucleoli are generally outstanding.

Two types of giant cells occur in cultures. The more common of them is simply a multinucleated form in which the nuclei are similar in size and other characteristics to those of the ordinary mononuclear cells. Binucleated cells are the most frequent (figs. 9, 11, and 16), but occasional cells may contain as many as 10 nuclei.

The second type of giant cell is relatively rare. In this type there may be 1 or several nuclei, but each nucleus is gigantic, with a diameter up to 2 or 3 times that of the majority of the nuclear population (fig. 12). Such nuclei usually contain proportionately large nucleoli, which in Wright's-stained preparations are pure blue. The cytoplasm of these cells is also increased in volume, but retains the vacuolation and staining characteristics of the general population. It is our impression that this

type of giant cell has increased in relative frequency in the later culture transfers. Such cells were not observed in smears of ascites from the early transfers in animals.

The cytoplasm of P329 cells in culture displays rather characteristic features. In hematoxylin-and-eosin-stained preparations, it is eosinophilic throughout, but there is usually a more intensely eosinophilic zone eccentrically placed around the nucleus (fig. 16). In multinucleated cells, the several nuclei usually appear enclosed within a single, more intensely eosinophilic zone. This eosinophilic cytoplasmic zone appears to be finely granular, and may occupy more than half the cytoplasmic area. Just outside the nuclear membrane one can sometimes distinguish a pale, crescentic zone suggestive of a "negative Golgi image," or hof. Peripheral to the eosinophilic central area, the cytoplasm usually appears foamy and vacuolated. Blunt-tipped pseudopodia are often noted, which also have a finely vacuolated structure except for the most distal portions, which are clear (hyaloplasm). In gelatin-sponge matrix cultures, P329 cells are generally "rounded up" and have a distinctly epithelioid appearance (fig. 13).

Coverslip cultures stained for fat with Oil Red O showed that the vacuolated, peripheral portions of the cytoplasm in the majority of the cells were filled with fat droplets, while the centrospheral (eosinophilic) areas contained none (fig. 9). The reciprocal of this picture was obtained when cultures were stained with the periodic acid-Schiff method after diastase treatment without preliminary dehydration through alcohol or xylol. In such preparations the centrospheral area stained strongly with the developed dye, while the peripheral cytoplasm was practically unstained (fig. 10). The composite picture provided by these methods is very similar to that described by Weiss and Fawcett (*17*) for normal chicken monocytes *in vitro*.

Phagocytic properties.—P329 cells in cultures showed a remarkable capacity for phagocytosis. When cultures were fed medium containing carbon black [6] in a proportion of 1.0 mg. per 5 ml., the cytoplasm of the majority of cells became filled with carbon in 24 hours (fig. 15). This was in sharp contrast, for example, with cells of the D189 human fibroblast strain, which showed practically no uptake of carbon under identical conditions.

Similarly, when P329 cultures were exposed to suspensions of heat-killed tubercle bacilli, the cells collected around small clumps of bacilli to form roughly spherical aggregates reminiscent of miliary tubercles. Acid-fast stains of individual cells on the coverslips adjacent to these aggregates showed numerous phagocytized bacilli within the cytoplasm (fig. 14).

In cultures accidentally contaminated with yeastlike organisms, P329 cells have been found gorged with the organisms. Their phagocytic activity did not appear to be significantly protective, however, since all cultures in which this contamination was recognized failed to survive.

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[6] Obtained from Godfrey L. Cabot, Inc., 77 Franklin St., Boston 10, Mass.

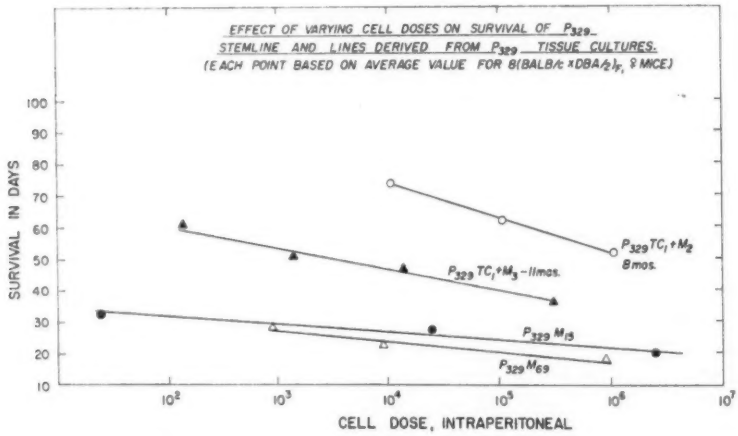
Biologic Behavior

Mouse inoculation.—Mouse inoculation of cultivated P329 cells was first attempted 5 months after the original isolation. At that time, because of the small culture population, only a small number of cells, estimated to be less than 100, was removed by teasing away a fragment of the still-remaining gelatin. This was inoculated intraperitoneally into a female (BALB/c × DBA/2)F_1 mouse. In a little less than 2 months, ascites developed. At autopsy this mouse showed the gross and microscopic features characteristic of mice that received cell transplants of P329. At 7 and 8 months, respectively, after isolation, doses of approximately 100,000 cells were inoculated intraperitoneally into groups of 3 (BALB/c × DBA/2)F_1 mice. All mice eventually developed P329 ascites tumors, and the animals not killed for autopsy died with massive neoplasms. However, 3 months elapsed before the ascites appeared in the mice inoculated with 7-month cultures, and 4 months before ascites developed in those inoculated with 8-month cultures.

At 11 and 12 months after isolation, million-cell doses of P329 culture cells were inoculated into groups of 8 and 6 (BALB/c× DBA/2)F_1 females, respectively. After 4 months of observation no tumors appeared, and it was thought that the inoculations had failed. However, the mice were kept and finally, 9 months after inoculation with an 11-month culture, 1 animal (age 10.5 months) developed ascites. A sample of ascitic fluid was removed and used for smears, for transfers to other (BALB/c × DBA/2)F_1 mice, for new cultures, and for a study of ploidy. Four days later, after the ascites had continued to increase, the mouse was autopsied. Grossly, the findings were typical of the P329 ascites tumor, consisting of an enlarged and nodular liver and spleen, enlarged intra-abdominal lymph nodes, bilaterally enlarged ovaries, and massive tumor deposits in the mesentery and gastric omentum. A little over 3.0 ml. of slightly bloody ascites was found. Wright's-stained smears of this fluid showed a cell population which differed in some respects from the original P329 tumor. The neoplastic cells still showed some of the characteristics of histiocytes but were generally larger and more pleomorphic than the original P329 strain. The cytoplasm was more basophilic and less vacuolated than before, and nuclei were larger and showed more frequent lobulated and doughnut-shaped forms. Multinucleated cells were more numerous and included many bizarre, monstrous forms. Histologic sections revealed comparable characteristics (figs. 3 and 6).

Cultures were set up simply by making a 1 : 300 dilution of the ascitic fluid from this mouse in the standard medium and introducing 1.0 ml. into Leighton tubes. The resulting growth showed the characteristic behavior of P329. The cells adhered tightly to the glass, enlarged, and became actively amoeboid within 48 hours. These cultures have been maintained for 4 months at the time of this report. Three (BALB/c × DBA/2)F_1 mice inoculated with 0.3 ml. of undiluted ascitic fluid from the mouse that yielded the neoplasm from the 11-month culture strain all developed de-

tectable ascites tumors 21 days after inoculation. At the third mouse passage, a survival-time/cell-dose relationship was determined for comparison with similar data obtained: *a*) for the original tumor at 15th mouse passage; *b*) for the 2nd serial mouse passage of an 8-month culture; and *c*) for the 69th consecutive mouse passage of the P329 stem line. The results are shown in text-figure 2. It is readily apparent that the ascites neoplasms derived from the 8- and 11-month cultures killed less rapidly than the P329 stem line at either the 15th or 69th passage.



TEXT-FIGURE 2.—Survival times of mice inoculated with graded cell doses of P329 from serially mouse-transferred line (P329 M15 and P329 M69) and from continuous culture line (P329 TC_1+M3—11 months and P329 TC_1+M2—8 months). P329 TC_1+M3—11 months designates third serial mouse passage of ascites tumor originating from 11-month culture. P329 TC_1+M2—8 months designates second serial mouse passage of ascites tumor originating from 8-month culture. Each point is based on an average value for 8 (BALB/c × DBA/2)F_1 female mice.

Host Specificity

To determine whether the host specificity of P329 cells carried in culture for 11 months had been altered, mice of the strains and crosses listed in table 1 were inoculated intraperitoneally with 10^6 cells from the third mouse passage of the 11-month culture strain. The results are presented in table 1, along with those obtained in the same strain and crosses when equal cell inoculums from the 62nd passage of the serially mouse-passed strain were used. No change of host specificity was discovered in either cell strain within the range of hosts tested. As might be expected, neither cell strain was capable of progressive growth in foreign hosts, including those with the same *H-2* histocompatibility allele (*H-2*^{d}) as the original DBA/2 host. Rejection of the transplants by the BALB/c and C57BL/10-*H-2*^{d} recipients was therefore brought about by histocompatibility differences outside the *H-2* influence. The identical pattern of host response to the 2 cell strains is confirmatory evidence of

the descent of the culture-derived tumor from the original P329 neoplasm, rather than from normal stromal cells that might have undergone neoplastic transformation *in vitro*. If the latter possibility were fact, the tumors originating from the culture strain should have grown progressively only in (BALB/c × DBA/2)F_1 hybrids, as this was the constitution of the host donating the explants to tissue culture.

TABLE 1.—*Transplantability characteristics of P329 culture—derived line compared with P329 stem line*

Host strain	*H-2* class	Third mouse passage of P329 cells after 11 months in culture	Sixty-second serial mouse passage of P329
		Fraction of mice dying with ascites tumors: Dose = 10^6 cells	
BALB/c	*H-2*^{d}	0/5	0/5
SWR*	Unknown	0/5	0/5
C57BL/Ka	*H-2*^{b}	0/5	0/5
C57BL/10-*H-2*^{d}	*H-2*^{d}	0/5	0/5
(C57BL/Ka × DBA/2)F_1	*H-2*^{b} × *H-2*^{d}	5/5	5/5
(BALB/c × DBA/2)F_1	*H-2*^{d} × *H-2*^{d}	5/5	5/5
DBA/2	*H-2*^{d}	5/5	5/5

*An inbred strain derived from Swiss mice, supplied by Dr. Margaret Deringer.

Chromosome Changes

When the morphologic changes described were noticed in the tumors derived from the 11-month culture strain of P329, it was suspected they might be associated with a change of ploidy. Using hypotonic salt methods to improve chromosome spreading, aceto-orcein squash preparations of ascites cells did not prove entirely satisfactory because of a tendency for the cytoplasm to rupture. Our observations at present are therefore limited to some of the most obvious differences between certain cell lines indicated later.

First the chromosomes were examined in ascites tumor cells from animals inoculated with substrains derived from culture cells. Classification of 200 metaphase figures into near-diploid and polyploid groups was performed on ascites tumor cells from the 11-month culture-derived strain in the first mouse passage, from the 8-month culture-derived strain in the 9th mouse passage, and from the 60th mouse passage of the stem line (never in culture). In point of time, these classifications were all performed during a 2-week period 21 months after the original tissue-culture isolation.

In the 11-month culture-derived strain, 198 of 200 figures fell in the polyploid range, and in the 8-month culture-derived strain, all the 200 figures were classified as polyploid. In both strains the great majority of polyploids was in the 4*n* range, with only occasional examples (2–4%) of higher ploidy in which 100 to 200 chromosomes could be counted.

In contrast, ascites cells from the 60th passage of the stem line showed only 7 percent polyploid mitoses among 200 classified. Two of the polyploid figures contained from 100 to 200 chromosomes, while the remaining 12 were near-tetraploid.

Chromosome counts were performed on 50 cells of the 11-month culture-derived strains in the 3rd mouse passage, and on 32 cells of the 69th passage of the stem line. In the former, counts ranged from 64 to 88, with a plateau of highest frequencies between 73 and 79, whereas in the latter, counts ranged from 40 to 48, with a mode of 42. A further outstanding difference between these 2 lines was the occurrence of as many as 5 metacentric chromosomes per cell in the 11-month culture-derived strain, whereas no metacentric chromosomes were observed in the stem line. Figures 17 to 19 illustrate examples from both lines.

From these data it might have been predicted that P329 cells growing *in vitro* would show a high degree of polyploidy comparable to that of the ascites tumors derived from 8- and 11-month cultures. This proved not to be true, however. Classification of 100 metaphase figures from cultures as late as 24 months after isolation showed 96 of these in the near-diploid category (fig. 20). The remaining 4 ranged from approximately tetraploid level to more than 200. From this finding, it is apparent that the high degree of polyploidy seen in the culture-derived ascites lines developed *after* introduction of culture cells into mice, rather than during their *in vitro* sojourn. It is, of course, quite possible that this polyploidy was accomplished through a process of selective proliferation of the polyploid portion of the culture population, subsequent to mouse inoculation.

Discussion

Perhaps the most interesting effect of cultivation upon this reticulum-cell sarcoma was the progressive alteration in tumor-producing capacity during passage in tissue culture. This change cannot at this time be considered a complete loss of tumor-producing ability, since neoplasms appeared in 2 animals inoculated with 11-month cultures, and in 1 animal inoculated with a 16-month culture. A loss of virulence for susceptible hosts, however, had developed, as shown by the long latent period after inoculation with long-term cultures, and by the long survival time of mice inoculated with large doses of ascites cells from subsequent mouse passages of the strains started from cultures. It seems better at present not to refer to these changes as a loss of neoplastic malignancy, since this term is ill-defined. To some workers, malignancy connotes primarily an intrinsic cellular change or changes, still poorly characterized, but hypothetically specific, which may be variable in degree. The basic changes in the malignant cell are only secondarily reflected by morphologic changes, unusual ability to proliferate, heterotopic existence, or survival after transplantation. To others, malignancy is defined purely in terms of performance under specific conditions, somewhat analogous to virulence as applied to infectious disease. Evaluation of the performance level necessarily takes into account interrelationships with the host, such as

inoculum size, site of inoculation, and genetic and metabolic host characteristics. Using the performance gauge, P329 declined in malignancy as a result of cultivation. However, if one accepts cellular atypia and polyploidy as reliable marks of malignancy, then P329 increased in malignancy during cultivation. The seeming discrepancy between morphologic features and biologic behavior of P329 reported here can be interpreted simply to show the dissociability of these 2 standards under certain highly artificial conditions. As Foulds (*18*) has indicated, the important objective is to identify the unit characters which alone, or in certain combinations, make cells able to behave in particular biologic patterns.

Several explanations can be offered for the decrease of virulence of P329 concomitant with its prolonged sojourn *in vitro*. Antigens acquired by the cells in culture might elicit an immunologic defense from the previously compatible hosts. Sanford *et al.* (*19*) have reported that NCTC clone 929 of the L strain of fibroblasts grown in tissue culture induced immunity to tumor formation by cells of this same strain when introduced to mice of the inbred strain (C3H) from which they originated. The heterologous serum in the medium conceivably might be a factor in producing this effect. Working with HeLa cells, Vainio and Penttinen (*20*) found that tissue-culture cells were more sensitive to the effects of antiserum when grown in heterologous (rabbit) serum than when grown in homologous (human) serum. The recent work of Sanford and coworkers (*21*) with cloned culture strains of high and low sarcoma-producing cells showed, however, that immunity induced by injection of whole cultures could not be duplicated by injection of the heterologous medium alone.

On the other hand, it is possible that in making the adaptation for survival and growth *in vitro*, the neoplastic cells acquired metabolic mechanisms that were a hindrance when the cells were returned to their former *in vivo* environment. An attempt is now being made to determine whether return to tissue culture of ascites cells taken from the animal inoculated with cells from an 11-month culture will result in a strain that can grow successfully both *in vivo* and *in vitro*.

The possibility has not been overlooked that the strain ultimately obtained in tissue culture might be derived not from the neoplastic cells, but from non-neoplastic cells in the original explants, since it is known that "normal" cells in tissue culture can take on the properties of neoplastic cells (*22–25*). Against this possibility are the successively prolonged latent periods which correspond with the increasing lengths of time in continuous culture. This gradual prolongation indicates progressive alteration of the neoplastic cells in culture, and not a sudden all-or-none change. We have never observed a latent period exceeding 5 weeks with the P329 line carried continuously in the mouse, even when minimal tumor-producing doses of 10 to 100 cells were used (text-fig. 2).

The increased latent period and prolonged killing time that were noted after the neoplastic cells were exposed to tissue-culture conditions merit further consideration. These phenomena have not often been recognized

because many of the continuous tissue-culture strains under study are of human origin and cannot be returned to isologous human hosts. Also, cell dosage cannot be accurately measured when solid tumors of laboratory animals are used and ascites tumors have only recently become available and been continuously cultivated. However, the observations reported here are not without parallel in other investigations. Earle, Shelton, and Schilling (*26*) reported a progressive lowering of tumor-producing ability in each of 6 strains of fibroblasts over a 3-year period. De Bruyn (*7*) found that the MB-III cell, derived from a murine lymphosarcoma, was incapable of tumor production after only 2 to 3 months of continuous cultivation free of normal connective-tissue cells. The original neoplasm in de Bruyn's study had not been adapted to an ascites form. Kieler (*27*) reported the loss of leukemia-producing ability in F_1 hybrids by Line I leukemia cells after as little as 6 weeks *in vitro*. These same cultures, however, retained their ability to produce tumors in mice of the inbred line of origin.

These few examples raise questions of considerable significance. To what extent may neoplastic cells relinquish their proliferative powers in the natural host in order to survive in a different and initially unfavorable environment? An analogy with the phenomenon of "smooth" and "rough" forms of bacterial organisms suggests itself here, as it has long been recognized that bacterial pathogens may lose much of their virulence after continued growth in artificial media, converting, in many cases, from smooth to rough forms in the process.

The change of ploidy found in ascites tumors resulting from animal inoculation with long-term cultures of P329 holds special interest because this change was not present in equal degree in the cells of the long-term cultures themselves. This finding provides further indication that rather drastic adjustments may be required of cell populations "acclimatized" to *in vitro* conditions upon return to the natural host. The mechanism and significance of such changes are not at present understood. Levan (*28*) has suggested that development of a polyploid population in mammalian neoplasms may be analogous to the development of polyploid forms in plants living under the extremes of climatic conditions (arctic and desert). This concept holds that the evolution of a polyploid population furnishes a mechanism whereby somatic cells can survive under very diverse environmental conditions.

Because of its continued morphologic and functional similarity to the histiocyte, the P329 culture strain should prove useful in investigations of phagocytosis, intracellular parasitism, the role of the histiocyte in bile formation, and, perhaps, certain aspects of immune reactions. Many possible uses of this cell strain are suggested by the problems discussed in the recent symposium on the physiopathology of the reticuloendothelial system (*29*). Dunn (*15*) has reviewed the morphologic and biologic characteristics of histiocytic neoplasms similar to P329, and Lewis (*30*) has described the *in vitro* behavior of one of these neoplasms in short-term culture. Gorer (*31*) has reported the very interesting finding that

2 histiocytic neoplasms during serial mouse transfer were seen to undergo morphologic change toward neoplasms simulating lymphoblastic leukemia. Serial mouse transfer of P329 beyond the 2-year period included in the present report has been accompanied by cytologic changes which appear to represent repetition of the phenomena observed by Gorer. Further observations on this point are being made, particularly with regard to behavior of the "blastlike" cells in tissue culture.

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PLATES

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PLATE 72

FIGURE 1.—Nodule of histiocytic neoplasm P329 in spleen of mouse (9th transfer generation) from which cell cultures were originally isolated. *Note* epithelioid appearance of the neoplastic cells as contrasted to hematopoietic elements in *upper left* of field. Hematoxylin and eosin. × 260

FIGURE 2.—P329 in liver of same animal illustrated in figure 1. *Note* elongated and indented nuclei resembling those of normal histiocytes. Liver parenchyma in *lower left* corner. Hematoxylin and eosin. × 520

FIGURE 3.—P329 growing in mesentery of mouse inoculated with culture 11 months after isolation. Compare with figure 2, noting larger cells, multinucleated forms, and bizarre mitotic figure. Hematoxylin and eosin. × 520

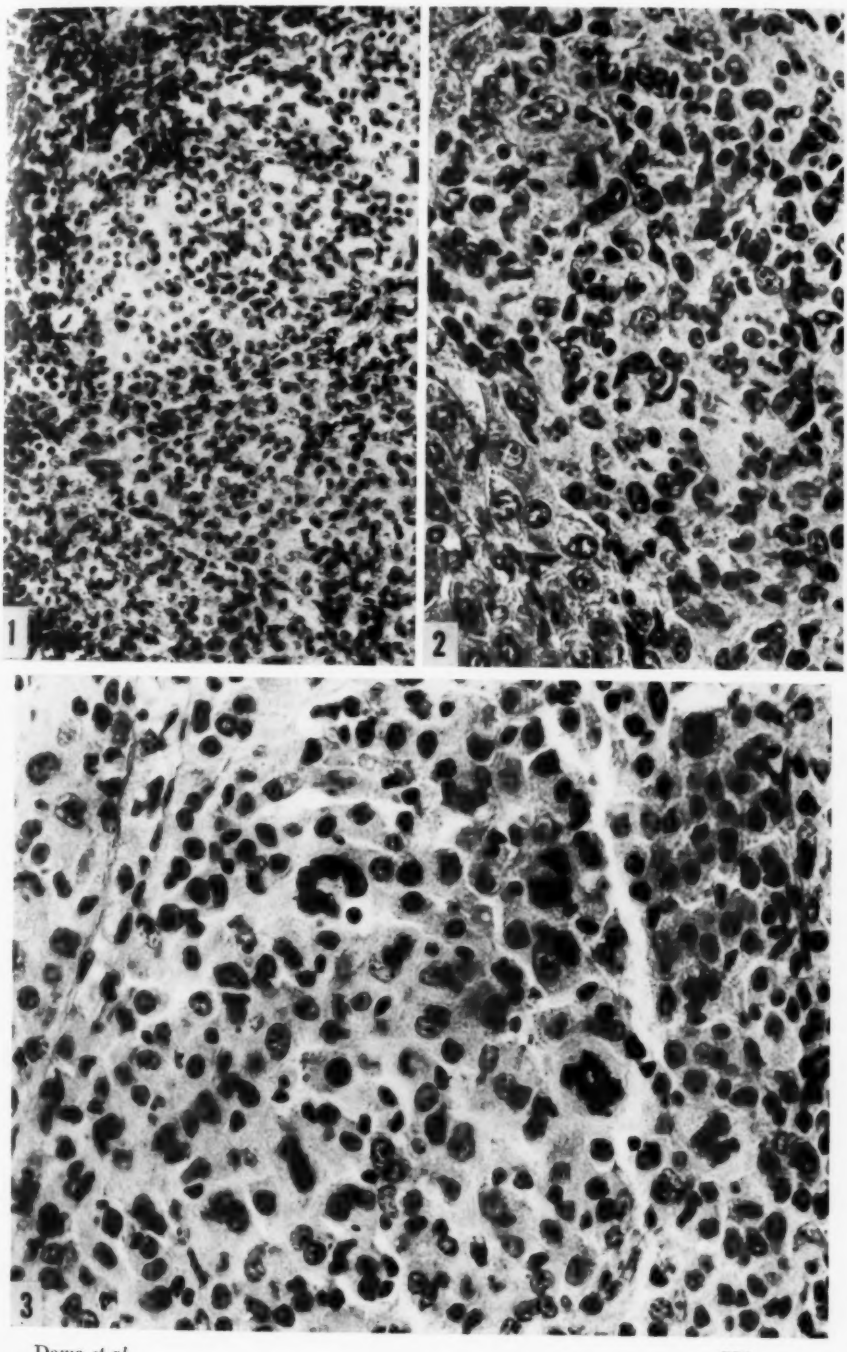

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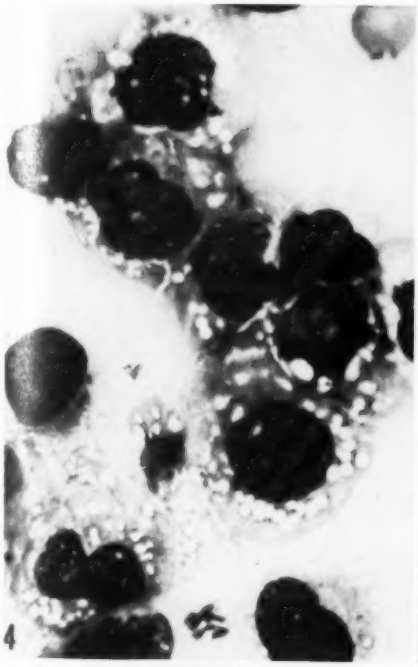
PLATE 73

FIGURE 4.—Smear of ascites from original mouse donor illustrated in figures 1 and 2. Cytoplasm is abundant and highly vacuolated, typical of histiocytic character. Wright's stain. × 1,000

FIGURE 5.—Smear of ascites from 70th consecutive mouse transfer of P329. Neoplastic cells still bear characteristics of histiocytes. A normal lymphocyte and 3 granulocytes are present in *left center*. Wright's stain. × 1,000

FIGURE 6.—Smear of ascites from mouse inoculated with P329 culture 11 months after isolation. Cytoplasm still shows some histiocytic characteristics, while nuclei show increased pleomorphism with many highly lobulated and ring forms as in *lower right*. Smear is from same animal illustrated in figure 3. Wright's stain. × 1,000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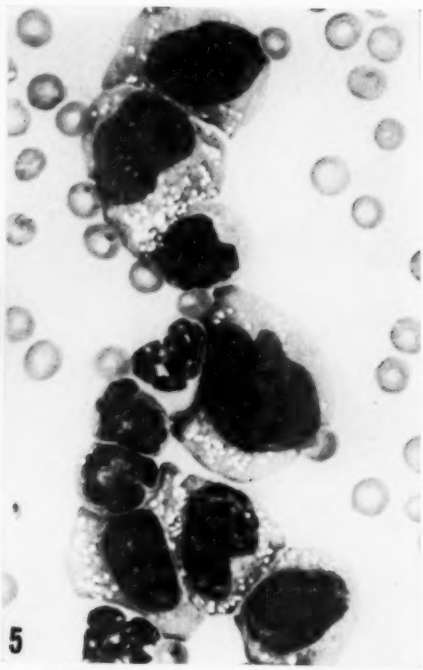
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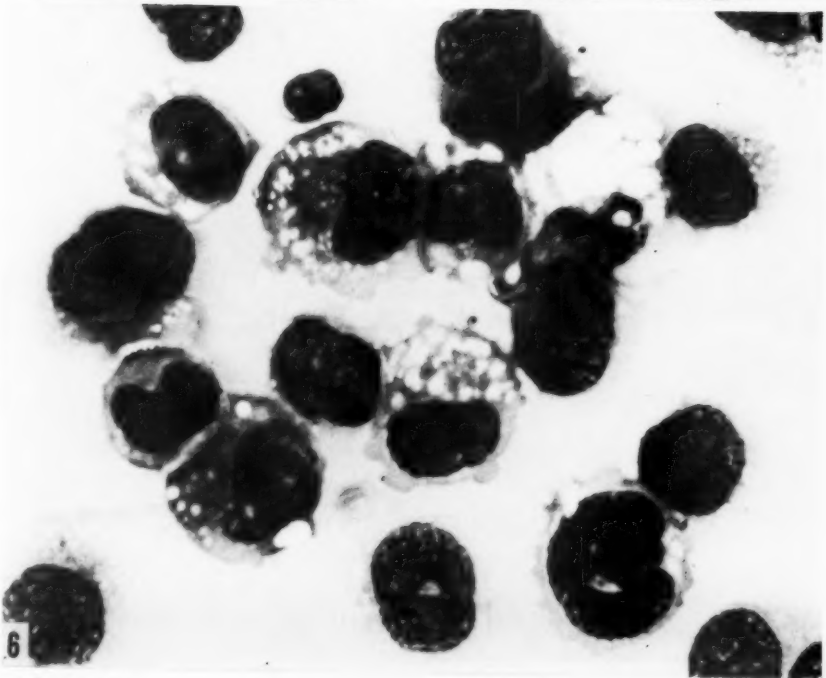
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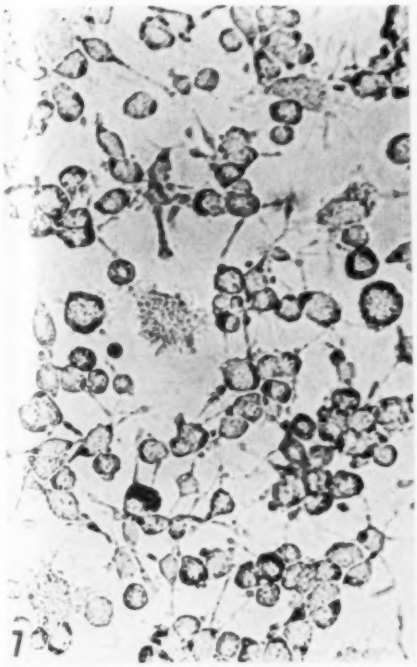
PLATE 74

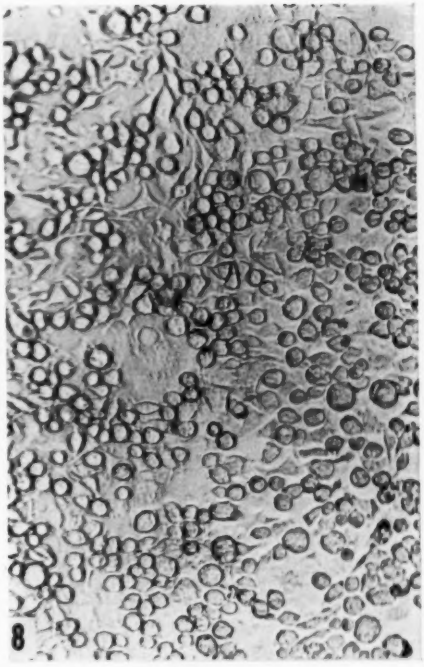
FIGURE 7.—Living culture of P329, 3 months after isolation, showing enlarged cells with numerous cytoplasmic processes, characteristic of P329 at this interval after explantation. Photograph is from culture strain P329 TC_2 (*11*) (*see* text-fig. 1), but is representative of appearance of the original P329 TC_1 strain at a comparable time. × 165

FIGURE 8.—Living culture of P329 TC_1, after 24 months *in vitro*. Compare with figure 7, noting smaller size of cells, fewer cytoplasmic processes, and more numerous round forms. × 165

FIGURE 9.—Coverslip culture of P329 TC_1, fixed in formalin and stained with Oil Red O. *Note* large unstained centrospheral area surrounded by dark zones composed of lipide droplets. × 620

FIGURE 10.—Coverslip culture of P329 TC_1 stained by periodic acid-Schiff method after diastase digestion. Centrospheral areas show strongly positive PAS reaction. × 520





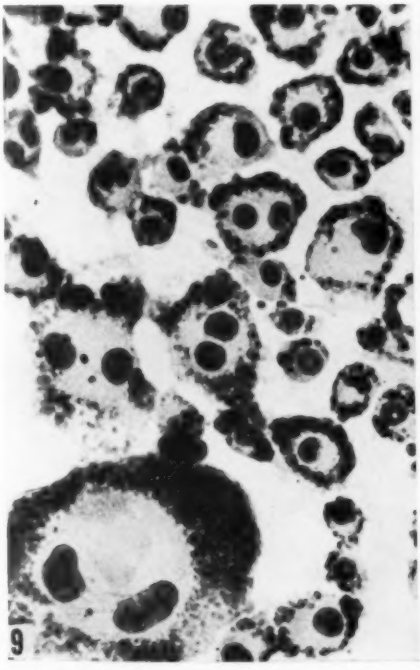


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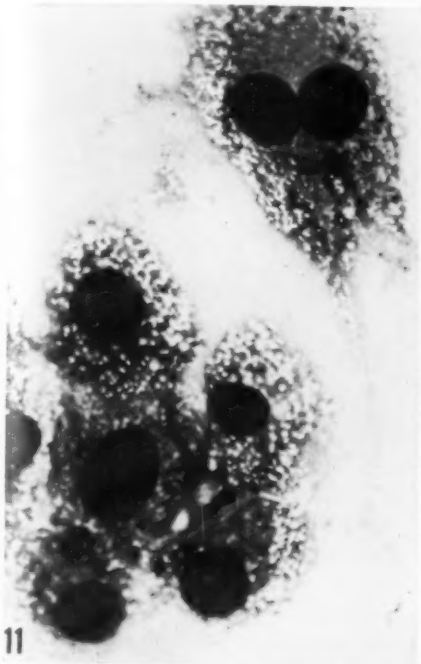
PLATE 75

FIGURE 11.—Smear of P329 TC_1 after 8 months in culture. Histiocytoid features of cells are readily apparent. Wright's stain. × 900

FIGURE 12.—Smear of P329 TC_1 after 24 months in culture. Histiocytoid features are still present. Giant cells of the type shown in lower half of the field have become more frequent as time has increased from original isolation. Nucleoli are visible as large, indistinct, pale areas at either pole of the giant nucleus. × 900

FIGURE 13.—P329 TC_1 growing in gelatin-sponge matrix culture. Cells grow well only on or near surface of sponge in such cultures. Some necrosis is evident in central part of this field. Hematoxylin and eosin. × 650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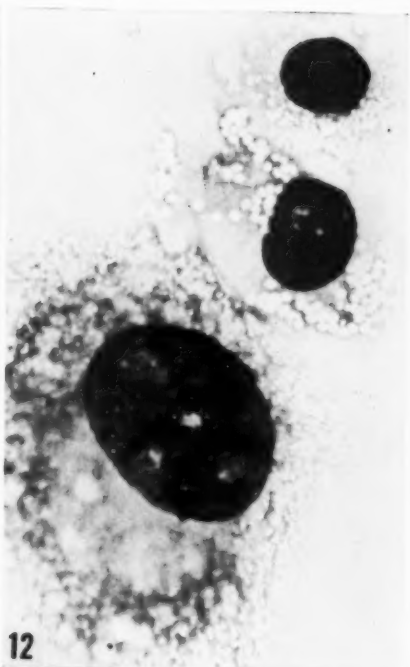
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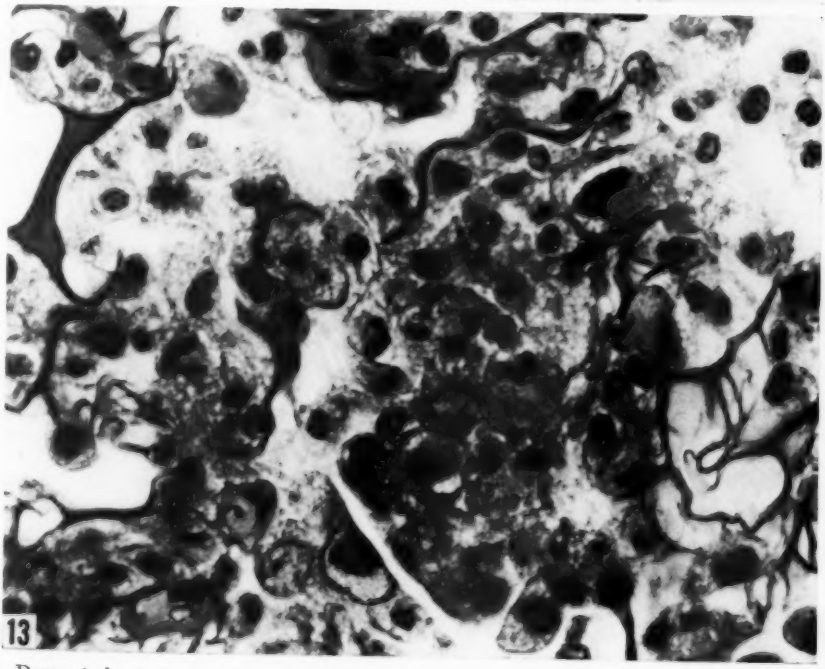
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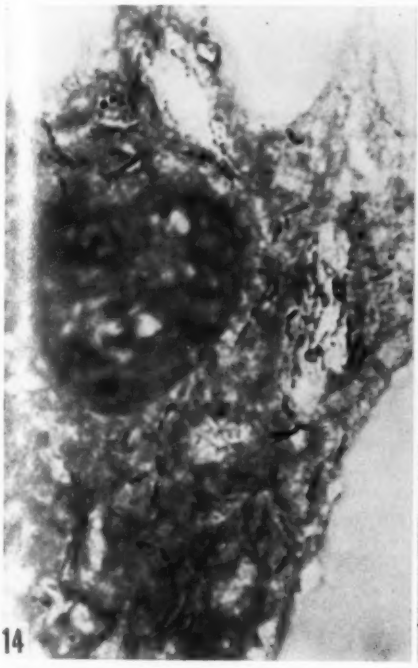
PLATE 76

FIGURE 14.—Cell from culture of P329 TC_1 exposed to suspension of killed tubercle bacilli for 24 hours, and stained by Ziehl-Neelsen method. *Note* phagocytized bacilli in cytoplasm. × 1,260

FIGURE 15.—Living culture of P329 TC_1 24 months after isolation, exposed for 24 hours to medium containing carbon black. Pale central areas represent nuclei surrounded by cytoplasm blackened with phagocytized carbon particles. × 165

FIGURE 16.—Wet-fixed coverslip culture of P329 TC_1 at 8 months. *Note* pleomorphism, with round, spindled, stellate, and binucleated forms. Compare with Wright's-stained smear in figure 11. Hematoxylin and eosin. × 900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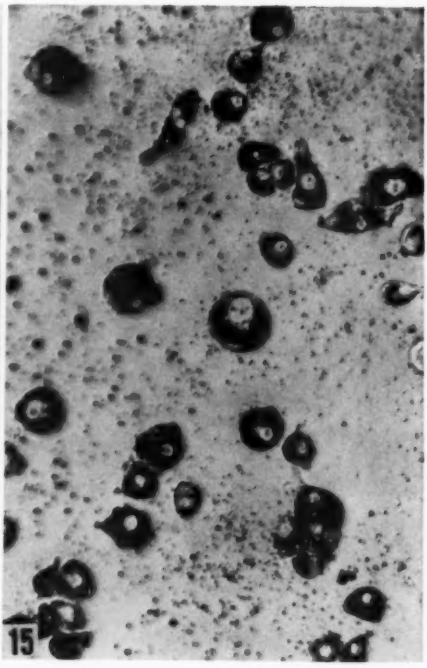
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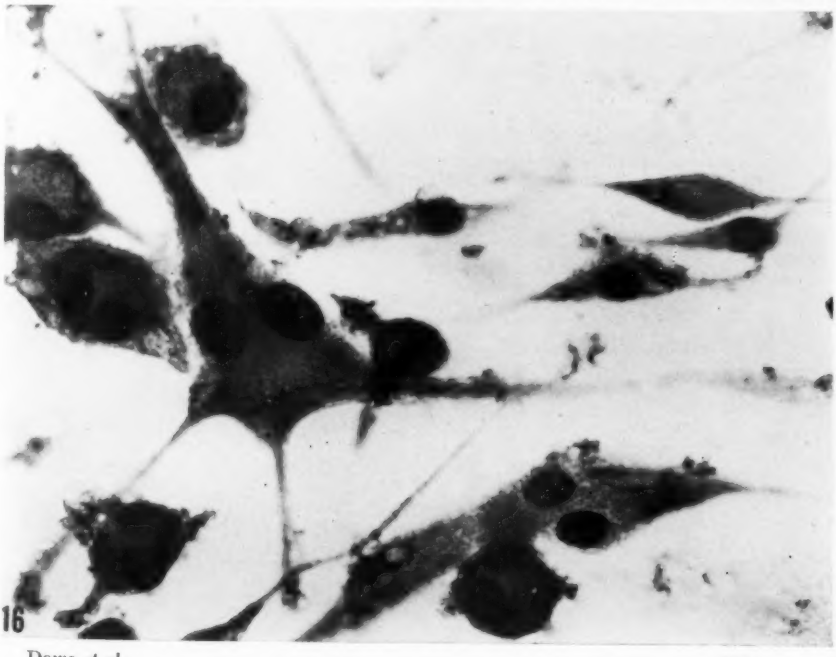
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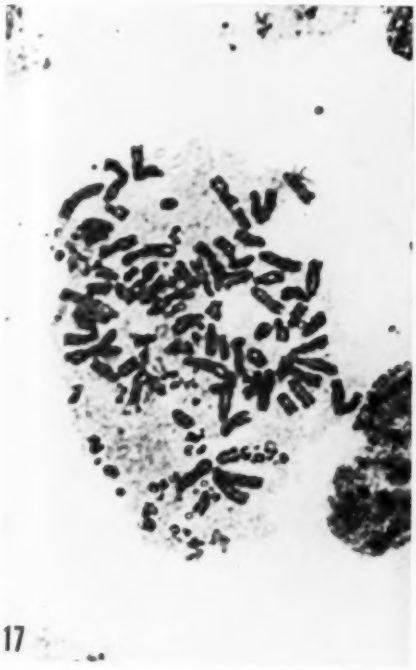
PLATE 77

FIGURE 17.—Aceto-orcein squash preparation of ascites from the third mouse passage of P329 after 11 months in culture. Ascites tumors derived from this 11-month culture were predominantly hypotetraploid. Seventy-two chromosomes were counted in this metaphase figure. Two metacentric chromosomes are identifiable at *top left*. Small, dark granular bodies are lipide droplets. × 1,400

FIGURE 18.—Aceto-orcein squash preparation of same material shown in figure 17. Three metacentric (M) and 2 subtelocentric (ST) chromosomes are clearly shown. × 1,600

FIGURE 19.—Aceto-orcein squash preparation of ascites from 69th serial mouse transfer of P329. This line was predominantly hyperdiploid. Cell shown here contained 42 chromosomes. Two chromosomes at *lower right* give illusory appearance of a metacentric. × 1,500

FIGURE 20.—Aceto-orcein squash preparation of P329 TC_1 culture, 24 months after isolation. Chromosome counts in cells from this source were predominantly near-diploid. The cell shown here contains 38 chromosomes, 2 of which are metacentric (M). × 1,600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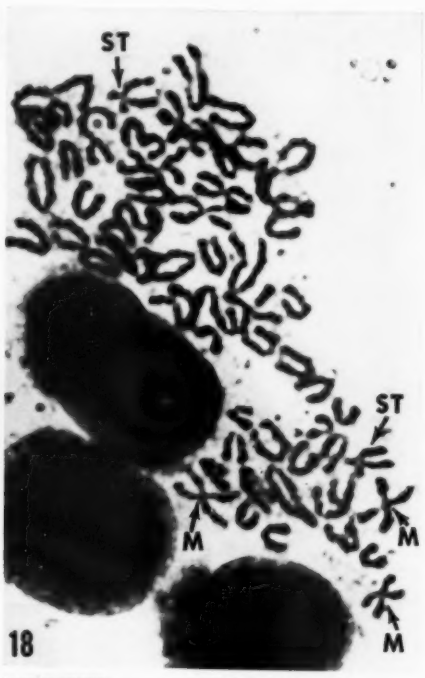
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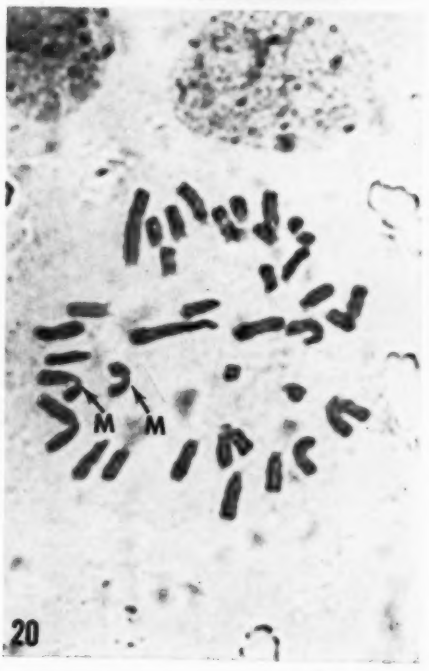
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Susceptibility of Agent-Free Inbred Mice and Their F_1 Hybrids to Estrogen-Induced Mammary Tumors [1]

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Summary

Agent-free mice of inbred strains C3H, BALB/c, RIII, DBA/2, I, and C57BL and their reciprocal F_1 hybrids were tested for susceptibility to mammary tumors induced by the subcutaneous implantation of diethylstilbestrol-cholesterol pellets. Strain C3H mice, (BALB/c × C3H)F_1 hybrids, and (RIII × C3H)F_1 hybrids were the most susceptible to the hormone-induced tumors. The high incidence of tumors in these 3 groups of mice makes them suitable animals for use in experiments in which it is desirable to procure mammary tumors in the absence of the mammary-tumor agent. The morphologic types of the mammary tumors in mice of the present series were similar to those previously described. The histogenesis of the tumors in the present experiment, especially those in old male mice receiving large doses of estrogen, was different since the tumors usually arose within a focus of altered fibrocystic mammary tissue. A glioblastoma multiforme was found in one of the treated mice. No previous record of such a neoplasm has been found except in mice receiving chemical carcinogens intracerebrally.—**J. Nat. Cancer Inst. 21: 783–811, 1958.**

This report records the results of 7 experiments in which mammary tumors occurred in mice that were free of the mammary-tumor agent and had received exogenous estrogenic stimulation. It is known that agent-free tumors can arise in mice; the pertinent literature has been reviewed by Dmochowski (*1*) and Mühlbock (*2*) and, more recently, by Boot and Mühlbock (*3*). The occurrence of mammary tumors in agent-free mice has received considerable attention in this laboratory since 1945 when it was found (*4*) that female F_1 hybrids procured from agent-free strain BALB/c females and strain C3H males showed a high incidence of breast tumors. A series of experiments (*5–10*) was performed to study the influences of heredity and the agent in the occurrence of these tumors, and the most recent paper (*10*) contained a summation of the studies. It was

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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concluded that the agent was not responsible for the high incidence of tumors in old hybrids but was involved in the production of tumors in a small number of young hybrids, and these latter tumors arose in mice that had acquired the agent from their fathers.

The investigations presented here stemmed from a desire to ascertain whether the father's agent could infect the F_1 hybrids directly while *in utero* or whether the mother was first infected and then transmitted the agent to her offspring through her milk. Because recognition of the agent in male mice is dependent upon the development of tumors and upon the proof that such tumors contain the agent, the young hybrid males were castrated and given diethylstilbestrol-cholesterol pellets. Mammary tumors began to appear when these males were only 5 months old and continued to occur until they were 21 months of age; the final incidence of tumors was 72 percent at a mean age of 12 months.

This experiment led to another in which mice of various agent-free inbred strains and their hybrids were tested for susceptibility to hormone-induced tumors. Of the remaining 5 experiments: 2 were designed to ascertain the incidence of mammary tumors in F_1 hybrids procured by mating BALB/c females to C3H+ males; 1 was undertaken to determine the susceptibility of agent-free C3H females to hormone-induced tumors; 2 were part of a series of studies on induced testicular tumors in mice of inbred strains and their hybrids. The last 2 experiments are included to compare the susceptibilities of intact and castrate males to hormone-induced mammary tumors.

It is important to mention that only tumors occurring in agent-free mice are included because the purpose of this paper is to inform the reader as to the relative susceptibilities of the inbred strains and their F_1 hybrids to hormone-induced mammary tumors.

Materials and Methods

All mice were raised in this laboratory. They were maintained under similar environmental conditions and given an unlimited supply of Purina laboratory chow and tap water. The only agent-containing mice used were strain C3H males that fathered F_1 hybrids in 3 experiments. These C3H males are identified by a "+" sign throughout this paper. All other mice were presumably agent free. The derivations of the strains and agent-free lines from high mammary tumor strains have been described in earlier publications (*5*, *11*, *12*).

Diethylstilbestrol was used to provide hormonal stimulation. Pellets containing 2.5, 5, 10, or 20 percent diethylstilbestrol dissolved in cholesterol were prepared according to the technique described by Shimkin and White (*13*). Each pellet weighed about 5 mg. and was inserted subcutaneously into the right axillary region by means of a 12-gauge trocar.

The tumors were fixed in Fekete's modification of Tellyesniczky's fluid (70 percent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part), embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Experimental Procedures and Results

In order to present the data briefly, the results of all 7 experiments are summarized in 4 tables in which the findings are not listed in the same order as the experiments are presented, but, in conformity with the purpose of this paper, according to the responses of the experimental animals and their genetic derivations. It is hoped this method of tabulation will be of assistance to those who are interested in the susceptibility of a particular strain or F_1 hybrid to the induction of agent-free mammary tumors. Table 1 includes groups of animals showing tumor incidences of more than 10 percent; table 2, those showing incidences of less than 10 percent; table 3, those that did not develop any mammary tumors. Table 4 summarizes the histologic findings in most groups showing incidences of more than 10 percent. Included also is a table summarizing the predominating types of tumors, other than those of mammary-gland origin, occurring in mice of the second experiment.

Experiment 1

As stated previously, this experiment supplied the first indication of the susceptibility of castrate male mice to hormone-induced agent-free mammary tumors. Strain BALB/c females were mated to strain C3H+ males and the resultant F_1 hybrids kept under observation for the occurrence of mammary tumors. Those appearing in females are not included because the females did not receive any exogenous hormonal stimulation.

Each hybrid male was castrated when 2 months old and, within the next 24 to 48 hours, received a 20 percent diethylstilbestrol-cholesterol pellet. The first tumor arose in a 5-month-old male and the last in one that was 21 months of age. Eleven that developed tumors at an early age were tested for the presence of the agent by the inoculation of tumor extracts or pieces of spleens into suitable test animals; none of the test animals developed a mammary tumor. These tests, together with the tumor histories of female litter mates, indicated that the agent was not involved in the production of tumors in the males.

Of 149 (BALB/c $\times$ C3H+)F_1 hybrid males, 108, or 72 percent, developed mammary tumors at a mean age of 12 months (table 1). This result showed that a high incidence of mammary tumors can be induced in agent-free castrate (BALB/c $\times$ C3H+)F_1 hybrid males by the administration of diethylstilbestrol-cholesterol pellets.

Experiment 2

This investigation was begun while the first experiment was in progress and after it was known that diethylstilbestrol was effective in eliciting mammary tumors in agent-free male mice. The purpose was to ascertain the relative susceptibilities of inbred strains and their reciprocal F_1 hybrids to the induction of agent-free tumors.

Six inbred strains that were devoid of the agent (C3H, BALB/c, RIII, DBA/2, I, and C57BL) and 12 groups of hybrids were used. Four strains

(C3H, BALB/c, RIII, and DBA/2) were susceptible to the agent and the remainder (I and C57BL) were resistant. Males were castrated when 2 months of age, and 24 hours later they and their female litter mates received pellets. Males were given 20 percent and females 2.5 or 5 percent diethylstilbestrol pellets. All mice surviving to the age of 24 months were killed and autopsied. A rather large number of mice were included in this experiment and the findings are presented in some detail.

The results in the various groups are distributed in tables 1, 2, and 3. Strain C3H males were the only inbred strain of animals with a tumor incidence of more than 10 percent, and strain C3H females the only inbred strain with an incidence between 0 and 10 percent. All other inbred strains of mice failed to develop mammary tumors. Obviously, strain C3H was most susceptible but the results do not permit an evaluation of the relative susceptibilities of the other 5 strains.

Strain BALB/c mice of both sexes were decidedly resistant to the hormone-induced growths for, as seen in table 3, none of 27 castrate males and none of 53 females developed tumors though they lived to mean ages of 16 and 21 months, respectively. All the remaining strains exhibited some degree of inability to tolerate diethylstilbestrol-cholesterol pellets; strain RIII males were very intolerant because castrates died within a few months after exposure to 20 percent diethylstilbestrol pellets and, as shown in table 3, strain RIII males were also the most intolerant of all mice to 10 percent pellets. Of the 41 RIII males listed in the table as receiving 10 percent pellets, 16 were from this experiment and these died at an average age of 16 months. RIII females were not used because a sufficient number was not available when the experiment was begun. Strain DBA/2 males were also susceptible to the toxic effects of diethylstilbestrol but not to such a degree that their resistance to hormone-induced tumors could not be established. Of the 88 males in table 3 that received 10 or 20 percent diethylstilbestrol pellets, 30 belonged in this experiment, received 20 percent pellets, and lived to a mean age of 19 months. Data in table 3 also show that strains I and C57BL animals of both sexes died at relatively early ages after receiving diethylstilbestrol pellets, but it is believed they survived long enough to show that they failed to approach strain C3H in susceptibility to estrogen-induced breast tumors.

Data in the 3 tables show that many reciprocal F_1 hybrid groups tolerated diethylstilbestrol better than did their inbred parents. Only 3 groups, males of BALB/c $\times$ I, BALB/c $\times$ C57BL, and I $\times$ C57BL origins, failed to live to an average age of at least 20 months. In table 3 the BALB/c $\times$ I males are combined with mice from experiment 7; of the 82 mice listed, 45 were castrate males from this experiment and these lived to a mean age of 19 months. The BALB/c $\times$ C57BL males of this experiment are also combined with mice of experiment 7. Of the 73 mice in this group, 35 were from this experiment and these lived to an average age of 21 months. Thus, the low average age for the entire group was caused by early deaths of mice of experiment 7.

TABLE 1.—*Mice with incidences of more than 10 percent mammary tumors following subcutaneous implantation of diethylstilbestrol-cholesterol pellets*

Expt. No.	Derivation of mice	Sex	Diethyl-stilbestrol in 5 mg. pellet (*percent*)	Number of mice	Number developing mammary tumor	Incidence (*percent*)	Average age (*months*)	Number without mammary tumor at death	Average age at death (*months*)
2	C3H	Castrate males	20	50	27	54	17	23	19
6	C3H	Intact males	20	32	4	12	19	28	20
1	BALB/c × C3H+*	Castrate males	20	149	108	72	12	41	15
2†	BALB/c × C3H	" "	20	97	57	59	14	40	19
7	" "	Intact males	20	27	10	37	12	17‡	12
3	BALB/c × C3H+	Castrate males	10	52	40	77	15	12	23
5	" "	" "	10	184	117	64	16	67	23
4	C3H	Virgin females	20	37	7	19	18	30	22
4	"	" "	10	41	5	12	20	36	24
2	BALB/c × C3H	" "	5	56	12	21	16	44	23
3	BALB/c × C3H+	Breeding females	5	111	46	41	21	65	23
5	" "	" "	5	226	94	42	22	132	24
2	RIII × C3H	Castrate males	20	25	21	84	13	4	15
2	" "	Virgin females	5	16	4	25	16	12	24
2	DBA/2 × C3H	Castrate males	20	45	7	16	19	38	21
2	BALB/c × RIII	" "	10	45	5	11	14	40	20

*In all tables a + sign denotes agent carrying strain C3H males. All other mice were agent free.

† In all tables hybrid data from experiment 2 include reciprocal hybrids.

‡ Ten developed testicular tumors at an average age of 10 months.

TABLE 2.—*Mice with incidences of less than 10 percent mammary tumors following subcutaneous implantation of diethylstilbestrol-cholesterol pellets*

Expt. No.	Derivation of mice	Sex	Diethyl-stilbestrol in 5 mg. pellet (*percent*)	Number of mice	Number developing mammary tumor	Incidence (*percent*)	Average age (*months*)	Number without mammary tumor at death	Average age at death (*months*)
2	C3H	Virgin females	5	25	1	4	17	24	23
2	"	" "	2.5	25	1	4	22	24	23
2	BALB/c × C3H	" "	2.5	41	3	7	23	38	24
2	DBA/2 × C3H	" "	5	39	2	8	20	37	23
2	" "	" "	2.5	29	1	3	19	28	23
2	I × C3H	Castrate males	20	26	1	4	18	25	20
2	" "	Virgin females	5	20	1	5	21	19	23
2	BALB/c × RIII	" "	5	56	1	2	13	55	22
2	" "	" "	2.5	44	1	2	23	43	23
7	" "	Intact males	20	37	2	5	13	35	12
2	BALB/c × DBA/2	Castrate males	20	49	3	6	19	46	21
7	" "	Intact males	20	24	1	4	12	23*	11
2	" "	Virgin females	5	44	1	2	19	43	23
2	" "	" "	2.5	41	2	5	22	39	23
2	DBA/2 × I	Castrate males	20	24	1	4	19	23	20

*Sixteen developed testicular tumors at an average age of 11 months.

TABLE 3.—*Mice that did not develop mammary tumors following subcutaneous implantation of diethylstilbestrol-cholesterol pellets*

Expt. No.	Derivation of mice	Sex	Diethylstilbestrol in 5 mg. pellet (*percent*)	Number of mice	Average age at death (*months*)
2	I × C3H	Virgin females	2.5	15	20
2	C57BL × C3H	Castrate males	20	30	22
2*	" "	Virgin females	2.5 or 5	35	23
2,6†	BALB/c	Castrate or intact males	20	94‡	11
2	"	Virgin females	2.5 or 5	53	21
2	BALB/c × RIII	Castrate males	20	15	19
2,7	BALB/c × I	Castrate or intact males	20	82	17
2	" "	Virgin females	2.5 or 5	34	20
2,7	BALB/c × C57BL	Castrate or intact males	20	73	17
2	" "	Virgin females	2.5 or 5	57	22
2,6	RIII	Castrate or intact males	10	41	10
6	"	Intact males	20	20	9
2,6	DBA/2	Castrate or intact males	10 or 20	88	16
2	"	Virgin females	2.5 or 5	33	21
2	DBA/2 × I	Virgin females	2.5 or 5	32	22
2	DBA/2 × C57BL	Castrate males	10	26	22
2	" "	Virgin females	2.5 or 5	51	22
2,6	I	Castrate or intact males	20	37	12
2	"	Virgin females	2.5 or 5	65	15
2	I × C57BL	Castrate males	20	51	19
2	" "	Virgin females	2.5 or 5	52	21
2,6	C57BL	Castrate or intact males	20	58	16
6	"	Intact males	10	31	19
2	"	Virgin females	2.5 or 5	59	17

*The results are combined when females of experiment 2, which carried 2.5 percent or 5 percent pellets, did not develop tumors.

†The results are combined when castrate males of experiment 2 and intact males of experiments 6 or 7 did not develop tumors.

‡Sixty-seven were intact males of which 51 developed testicular tumors at an average age of 10 months; 27 castrates died at a mean age of 16 months.

Six groups of F_1 hybrids showed mammary-tumor incidences of more than 10 percent. An incidence of 59 percent in BALB/c × C3H castrate males confirmed the findings of the first experiment. An incidence of 21 percent in BALB/c × C3H females that had received 5 percent diethylstilbestrol pellets showed that females of this hybrid derivation will also develop tumors in response to exogenous hormonal stimulation, and supported the results with castrate males. Indeed, throughout the experiment, tumor incidences in females did, in general, confirm the susceptibility of their male litter mates that received considerably more hormonal stimulation.

In this experiment the only discrepancy deserving comment was the occurrence of breast tumors in 5 of 45 BALB/c × RIII males that received 10 percent diethylstilbestrol pellets (table 1) and in none of 15 that received 20 percent pellets (table 3). This difference cannot be

attributed to the average age of survival or to the occurrence of other types of tumors in those carrying 20 percent pellets.

A tumor incidence of 84 percent at a mean age of 13 months in RIII × C3H males was of special interest for it showed that mice of this hybrid cross are also excellent animals for studies of agent-free mammary tumors. Further work is necessary to determine the relative susceptibilities of BALB/c × C3H and RIII × C3H hybrids. Richardson (*14*) has reported recently that mammary tumors did not appear in 91 female and 90 intact male (C57L × RIII)F_1 hybrids that received 20 percent diethylstilbestrol pellets when 8 weeks of age.

Two results of over-all interest were obtained in this experiment: 1) Strain C3H mice and F_1 hybrids from them were established as suitable test animals for the acquisition of agent-free mammary tumors. 2) In accordance with observations recorded in an earlier paper (*5*) dealing with the occurrence of mammary tumors in agent-free females, those strains most susceptible to the agent-induced tumors produced F_1 hybrids that were most susceptible to the occurrence of agent-free tumors. The following paragraph is taken from the discussion of the earlier paper.

"Analysis of the data showed that most of the tumors appeared in hybrid descendants of strains C3H—, dba—, and C—, which not only possess a predisposition to the development of mammary tumors, but which, in the presence of the mammary-tumor agent, are high-tumor strains. Thus, there was a parallelism between an inherited tendency to mammary-tumor development and susceptibility to the mammary-tumor agent. If those strains of inbred mice possessing an inherited predisposition to the development of mammary tumors are also susceptible to the mammary-tumor agent, then the role of the agent in the production of tumors could be that of an accelerator which markedly increases the incidence of tumors within the life spans of the mice."

The present experiment extends this suggestion, derived from an investigation including strains C3H, DBA/2, and BALB/c, to include strain RIII that in the presence of the mammary-tumor agent is also a high-tumor strain.

Experiment 3

This experiment was similar to the first. Strain BALB/c females were mated to C3H+ males and each raised from 3 to 5 litters. The F_1 hybrids thus produced were the test animals of this experiment. When they were 2 months old the males were castrated and 1 day later received 10 percent diethylstilbestrol pellets; the females were bred, permitted to raise 1 litter, and received 5 percent diethylstilbestrol pellets 1 month after their litters were weaned.

The occurrence of mammary tumors is shown in table 1. The incidence of tumors in males was 5 percent more and the average age when tumors appeared was 3 months later than in males of experiment 1 that were exposed to 20 percent diethylstilbestrol pellets. Thus, 10 percent diethylstilbestrol pellets were almost as effective in producing mammary tumors as those containing 20 percent of the estrogen.

The females showed a tumor incidence of 41 percent at a mean age of 21 months, which was considerably higher than a previously recorded incidence of 9 percent at an average age of 21 months in 46 BALB/c × C3H females that had given birth to 1 litter (*9*). Indeed, an incidence of 41 percent mammary tumors in BALB/c × C3H+ hybrid females of the present experiment compares favorably with incidences in similar females of other experiments (*9, 10*) that had given birth to 3 litters in rapid succession. It is clear that the administration of diethylstilbestrol pellets can supplant the time-consuming breeding procedure in producing mammary tumors in females of this hybrid derivation.

Experiment 4

The mice used in this experiment were 1.5 to 4.5 months old, nonbreeding strain C3H females that served as controls in an investigation involving agent-free mammary tumors. Two groups of controls were used; one received 10 percent and the other 20 percent diethylstilbestrol pellets.

The results are shown in table 1. Tumor incidences in both groups were higher than those in virgin females of experiment 2 that received 5 percent diethylstilbestrol pellets, but lower than the incidence in castrate males of experiment 2 that received 20 percent diethylstilbestrol pellets.

Thus, the findings in these strain C3H females indicated that higher concentrations of diethylstilbestrol could have been administered to the females of experiment 2.

Experiment 5

This investigation was undertaken because of the findings in experiment 3. The experimental procedure with (BALB/c × C3H+)F_1 hybrids was the same as in experiment 3, but more animals were procured. Males were castrated at 2 months of age and 1 day later given 10 percent diethystilbestrol pellets. Females were bred when 2 months old and given 5 percent diethylstilbestrol pellets 1 month after their litters were weaned.

The results of this experiment are shown in table 1 and call for little comment because the incidences of tumors in both sexes were similar to those of experiment 3. Hence, they emphasized the usefulness of (BALB/c × C3H) hybrids in studies of agent-free mammary tumors induced by estrogenic stimulation.

Experiment 6

This experiment was one of a series of investigations dealing with the production of testicular tumors by estrogenic stimulation. It is included here to compare the susceptibilities of intact and castrate male mice to estrogen-induced breast tumors.

In the experiment, 3- to 6-month-old agent-free males of strains C3H, BALB/c, RIII, DBA/2, I, and C57BL received diethylstilbestrol-cholestrol pellets. Those of strains C3H, BALB/c, and I were given 20 percent diethylstilbestrol pellets and those of strains RIII, DBA/2, and

C57BL, because of their intolerance to the estrogen, received 10 or 20 percent pellets.

Mammary tumors appeared in strain C3H males only and, as seen in table 1, only 12 percent developed tumors. Heston and Deringer (*15*) reported a mammary-tumor incidence of 20 percent in 60 agent-free strain C3H males that were 1 to 2 months of age when they received, subcutaneously, 10 percent diethylstilbestrol pellets weighing 5 to 8 mg. A tumor incidence of 12 percent in intact males of the present experiment was about the same as the incidence in virgin strain C3H females of experiment 4 that received 20 percent diethylstilbestrol pellets. This implies that castration did much to increase the susceptibility of strain C3H males. None of the other mice developed a breast tumor and they are, therefore, listed in table 3.

BALB/c mice are very susceptible to estrogen-induced testicular tumors and this fact makes it difficult to ascertain the susceptibility of intact males to estrogen-induced breast tumors. However, experiment 2 showed that castrate males were resistant. Moreover, many more intact males received 20 percent diethylstilbestrol pellets in other experiments (*16*) and less than 1 percent developed breast tumors. The intact strain RIII males were too intolerant to both 10 and 20 percent pellets to reveal their tendency toward the development of breast tumors. In this experiment, 25 received 10 percent pellets and died at an average age of 8 months. Strain DBA/2 lived to an average age of 14 months following insertion of 10 or 20 percent pellets and, since none developed a breast tumor, it is safe to assume they were more resistant than strain C3H. Strain I males received 20 percent pellets only and 15 used in this experiment died at a mean age of 10 months. Consequently, their degree of susceptibility to estrogen-induced breast tumors was not determined with this dose of hormonal stimulation. Strain C57BL males that received 10 percent pellets survived to an average age of 19 months, and 29 that received 20 percent pellets died at a mean age of 16 months. This permits the conclusion that these C57BL males were resistant to the development of breast tumors.

The results of this experiment confirmed the findings of experiment 2 in which castrate mice were used, namely, that strain 3CH males are satisfactory animals when one attempts to induce breast tumors in agent-free inbred strain males.

Experiment 7

The purpose of this experiment was to determine the susceptibilities of F_1 hybrid mice to induced testicular tumors. Strain BALB/c females were bred to agent-free males of strains C3H, RIII, DBA/2, I, and C57BL and, when the offspring were 2 months old, they received 20 percent diethylstilbestrol pellets. The experiment gave satisfactory results concerning susceptibility to testicular tumors because the hybrids were more tolerant of 20 percent diethylstilbestrol pellets than were the inbred strains of experiment 6. The experiment was terminated when surviving mice were between 17 and 18 months of age.

The occurrence of mammary tumors is shown in the first 3 tables. Only the BALB/c × C3H hybrids exhibited an incidence of more than 10 percent (table 1). Of 27 mice, 10, or 37 percent, developed mammary tumors despite the appearance of testicular tumors in 10 mice at an average age of 10 months; 2 mice developed both mammary tumors and testicular tumors. Two BALB/c × RIII and 1 BALB/c × DBA/2 hybrids were the only other mice to develop breast tumors (table 2).

The results of this experiment confirmed the susceptibility of BALB/c hybrids to estrogen-induced mammary tumors and, in addition, indicated that the F_1 hybrids were better test animals than their inbred parent strains of experiment 6 because of the greater tolerance of the hybrids to diethylstilbestrol. The only hybrids to show ill effects from diethylstilbestrol pellets were those derived from strains BALB/c and RIII. There were 37 of these mice and the average age of survival was 12 months (table 2). However, 9 died within 3 months after implantation of pellets; the remaining 26 that did not develop mammary tumors lived to a mean age of 16 months.

Morphology of the Tumors

The mammary tumors developing in mice of these experiments were sorted according to a scheme previously described. This divides mammary tumors into adenocarcinomas, types A, B, and C, adenoacanthomas, carcinosarcomas, and a miscellaneous group (*8*, *17*). Adenocarcinomas, types A and B, are composed predominantly of epithelial glandular structures. The glandular structures of the type A tumors consist principally of small spaces lined by cuboidal cells, resembling mammary acini. The type B tumors show greater diversity of structure. They may be composed of a variety of epithelial glandular patterns, such as solid sheets of cells, cystic papillary formations, cords of cells, or combinations of these. The amount of connective-tissue stroma in the type B tumors and the prominence of vascular spaces separating the epithelial elements vary greatly. Adenocarcinoma, type C, has a distinctive pattern. It is composed of small cysts lined by cuboidal epithelial cells, surrounded by an outer layer of fusiform cells resembling myoepithelium. Adenocarcinoma, type C, is a relatively rare type. The adenoacanthomas show a mingling of glandular and squamous-cell elements. The carcinosarcomas contain two neoplastic elements, one resembling epithelial cells and the other composed of fusiform cells resembling fibroblasts. The miscellaneous group of mammary-gland tumors of the mouse comprises a few rare and unusual forms not clearly belonging to any one of the other groups.

When large numbers of tumors from mice of different ages and inbred strains, with and without the mammary-tumor agent, have been sorted on the basis of their distinctive histologic characteristics, some degree of correlation has been found between the morphology of the tumors and the groups of mice in which the tumors arose. Type A tumors always predominated in young C3H mice with the agent, and types A or B

mammary tumors taken together were always most frequent in mice of other strains, such as BALB/c with the agent. Type C mammary tumors, especially common in hybrids with a strain BALB/c parent, were found most often in old mice without the agent. Adenoacanthomas were common in old strain C3H mice without the agent. Carcinosarcomas were noted frequently in strain DBA/2 mice that were free of the agent and had been painted with methylcholanthrene. Miscellaneous tumors appeared infrequently, most often in old mice without the agent.

The results of sorting the tumors from the present experiments are shown in table 4. Relatively few adenocarcinomas, type A, were found. This was in accord with our previous experience in mice without the agent. The types and distribution of the other tumors, however, did not follow closely the general pattern previously observed in agent-free mice, because adenocarcinomas, type C, and adenoacanthomas were less frequent. The mice that did develop tumors of these types were in the oldest age group, indicating that age may be an important factor in determining the histologic pattern of the mammary tumor. The only notable difference in tumor type among the groups listed in table 4 was between the castrate BALB/c $\times$ C3H males and the hybrid females of the same genetic constitution. Adenocarcinoma type C and adenoacanthomas were more common in the females than in males, possibly because the females reached a more advanced age.

In untreated mice of strain C3H and their hybrids, especially when the agent is present, mammary tumors usually arise in a "hyperplastic nodule," a collection of small glandular structures budding off from a duct (*18*, *19*). The fully developed tumors on gross examination are usually rather soft, and when cut across may reveal a number of large or small cysts filled with clear or bloody fluid. In contrast, many of the estrogen-induced tumors, especially those in the males, were firm and white, and when cut revealed cystic areas that in most tumors were full of a thin, milky fluid. Occasional tumors were soft or gelatinous and some contained a thick, gray or brown fluid.

Microscopic examination of many of the tumors explained the unusual findings on gross examination. A central mammary adenocarcinoma of the usual type A or B structure was often surrounded by, or encased in, tissue composed of small cysts of fairly uniform size lined by flat or low cuboidal epithelium. The cysts contained an eosinophilic substance that often formed concentric bodies resembling the corpora amylacea of the prostate. The cysts were separated by heavy bands of connective tissue so that a distinctive combination of fibrous tissue and small cysts was formed that will be referred to as "fibrocystic tissue" (figs. 1 through 6). This tissue did not involve all the normal mammary-gland area, for it was usually found in well-circumscribed masses, and the margins were often sharply outlined, suggesting that a focal lesion had developed in a limited area of breast tissue (figs. 2 and 3). In a few microscopic sections, nodules were seen that were composed entirely of the fibrocystic tissue, and these nodules were so compact and sharply circumscribed from the sur-

TABLE 4.—*Types of mammary tumors occurring in mice of table 1*

Expt. No.	Derivation of mice	Sex	Diethylstilbestrol in 5 mg. pellet (*percent*)	Number developing mammary tumor	Type A		Type B		Type C		Adenoacanthoma		Miscellaneous		Carcinosarcoma	
					Number of tumors	Average age (*months*)	Number of tumors	Average age (*months*)	Number of tumors	Average age (*months*)	Number of tumors	Average age (*months*)	Number of tumors	Average age (*months*)	Number of tumors	Average age (*months*)
2	C3H	Castrate males	20	27	7	15	20	18								
1	BALB/c × C3H+	Castrate males	20	108	17	15	90	12			1	10				
2	BALB/c × C3H	Castrate males	20	57	13	14	43	14	1	20						
7	BALB/c × C3H	Intact males	20	10	—	—	8	13								
4	C3H	Virgin females	20	7	4	19	2	12							1	24
4	C3H	Virgin females	10	5	3	19	2	22								
2	BALB/c × C3H	Virgin females	5	12	3	14	8	17			1	20				
5	BALB/c × C3H+	Breeding females	5	94	8	20	58	22	5	23	5	23	1	19	1	19
2	RIII × C3H	Castrate males	20	21	4	14	16	13			1	19				
2	RIII × C3H	Virgin females	5	4	2	17	2	15								
2	DBA/2 × C3H	Castrate males	20	7	—	—	7	19								
2	BALB/c × RIII	Castrate males	10	5	3	12	2	16								
Total				357	64	16	258	15	6	23	8	21	1	19	2	21

rounding adipose tissue that on gross examination they resembled tumor nodules of very firm texture. It was unusual, however, to have a nodule composed entirely of the fibrocystic tissue, because one to several foci of cellular mammary-tumor tissue of one of the usual glandular epithelial patterns could nearly always be found within the fibrocystic areas (figs. 2, 3, and 4). The cells of the tumors often contained secretory globules, probably the source of the milky fluid noted on gross examination (fig. 1). Apparently foci of mammary tumor with a vigorous growth capacity developed within the fibrocystic tissue, and compressed and replaced it. Even large tumors, however, usually had remnants of the fibrocystic tissue at the margins (fig. 1). The earliest formation of tumors sometimes appeared to arise from cells lining the cysts (figs. 4 and 5).

Besides the fibrocystic tissue composed of small uniform cysts, much larger cysts lined by a single layer of epithelial cells were found occasionally (fig. 6). The fibrocystic tissue was sometimes invasive as indicated by extensions into muscle and lymph nodes (fig. 6).

The fibrocystic tissue was noted most often in males that received high doses of estrogens. This tissue was least frequent in females and younger males. The only males in which this tissue was absent were under 10 months of age. Among the female mice, none showed a marked fibrocystic tissue reaction and only a few evidenced a slight reaction, while the large majority showed none. It would thus appear that a high dose of diethylstilbestrol and a prolonged period of activity were necessary to produce the fibrocystic tissue change in the mammary glands.

Occurrence of Other Tumors in Experiment 2

Although the primary aim of the second experiment was to ascertain the susceptibility of inbred strains and their reciprocal hybrids to the development of mammary tumors, the occurrence of other tumor types deserves attention because of the number of animals used, their known genetic constitutions, and their exposure to pellets containing different concentrations of diethylstilbestrol. The lack of untreated controls made it impossible to evaluate the role of estrogenic stimulation in the production of tumors except where there was a clear correlation between dose of hormone and incidence of a particular type of tumor. Gardner (*20*), Kirschbaum (*21*), and Shimkin (*22*) have written summaries of the role of hormones in the production of tumors in experimental animals.

To present the data as briefly as possible the results are summarized in table 5 which lists the predominating tumors found in these animals. Almost all the mice came to autopsy and it is essential to remember that all survivors were killed when 24 months of age. The last 2 columns of the table are included for the convenience of the reader, who need not refer to other tables for the number of mice that developed breast tumors and for the average age at death of those dying without breast tumors. Mean ages of tumor occurrence are not given but, with the exception of lymphocytic lesions, very few arose in mice less than 15 months old and many were found when the animals were killed at 24 months. Thus, the

occurrence of mammary tumors in certain groups could account for fewer tumors of other types.

Lymphocytic tumors were the most prevalent lesions. Of 53 groups of mice listed in the table only 6 were free of lymphocytic hyperplasia. Mice with these tumors are presented according to the sites of origin of the neoplasms. Almost all lesions confined to the thymus gland and some mesenteric lesions were diagnosed microscopically, but very few tissues from mice with generalized lesions were fixed because these mice displayed the characteristic signs of leukemia in this species.

Sixty-two mice developed enlarged thymus glands only and, of the inbred strains, BALB/c and I showed the highest incidences. These tumors had been seen previously in BALB/c males that had received 20 percent diethylstilbestrol pellets (*16*) but their occurrence in estrogen-treated strain I mice was a new observation. The 13 strain I females with enlarged thymus glands came to autopsy at a mean age of 13 months. Strain I mice have been maintained in this laboratory since 1935 and have shown a much lower incidence of leukemia than those of this experiment. Furthermore, lymphocytic lesions limited to the thymus were seldom found in untreated mice.

F_1 hybrids showing the highest incidence of hyperplastic thymic lesions belonged to the BALB/c × I, DBA/2 × I, and C57BL × I crosses, with the highest incidence in the BALB/c × I hybrids. Thus, the response of these hybrids was in conformity with the susceptibilities of their parent strains.

Forty-one mice died with enlarged mesenteric lymph nodes. These lesions were of no special interest with the exception of those in BALB/c × RIII hybrids; of the 41 mice, 15 were of this hybrid derivation.

Most mice developing general lymphocytic lesions showed the typical signs of leukemia: enlargement of lymph nodes, including mesenteric nodes and frequently the thymus gland, together with infiltration of organs, especially the spleen, liver, and kidneys. Data in the table show that of the inbred strains, mice of strains BALB/c and C57BL exhibited the highest incidences of 19 and 15 percent, respectively. In hybrids the following 3 groups showed highest incidences: DBA/2 × I, 18 percent; BALB/c × I, 18 percent; I × C57BL, 15 percent. It is clear that strain I exerted a strong influence upon the occurrence of generalized lesions.

When all lymphocytic lesions were used to compute tumor incidence, the 3 most susceptible strains were BALB/c, I, and C57BL, with incidence of about 30, 25, and 20 percent, respectively. Among the F_1 hybrids the 3 most susceptible were BALB/c × I, DBA/2 × I, and I × C57BL with incidences of about 37, 30, and 24 percent, respectively. Strain C3H mice and all their hybrids developed fewer lymphocytic lesions despite the fact that estrogenic stimulation induces leukemia in some C3H substrains (*20*). It is also seen that BALB/c × DBA/2, DBA/2 × C57BL, and BALB/c × C57BL hybrids were resistant, whereas all of their parent strains, when crossed with strain I, produced susceptible hybrids.

The table shows that 144 mice developed pulmonary tumors. These

TABLE 5.—*Occurrence of tumors in inbred mice and their reciprocal F_1 hybrids of experiment 2*

Derivation of mice	Sex	Diethylstilbestrol in 5 mg. pellet (*percent*)	Number of mice	Lymphocytic lesions			Pulmonary tumors	Hepatomas	Adrenal tumors	Pituitary tumors	Sarcomas	Hemangioendotheliomas	Mammary tumors	Average age at death without mammary tumors (*months*)
				Thymus	Mesentery	General								
C3H	M	20	50	—	—	1	6	6	—	—	2	—	27	19
"	F	5	25	—	—	—	2	3	—	—	—	—	1	23
"	F	2.5	25	1	1	2	—	7	—	—	2	—	1	23
BALB/c	M	20	27	6	—	6	3	—	1	—	—	—	—	16
"	F	5	27	—	1	6	8	—	3	—	1	1	—	21
"	F	2.5	26	—	1	3	4	—	1	—	1	1	—	21
DBA/2	M	20	30	1	—	1	—	—	—	—	—	—	—	19
"	M	10	8	—	—	1	—	—	—	—	—	—	—	21
"	F	5	22	1	—	3	2	—	—	—	2	—	—	21
"	F	2.5	11	—	1	—	1	—	—	—	—	—	—	21
I	M	20	22	3	—	1	—	—	1	—	—	—	—	14
"	F	5	34	7	1	2	1	—	—	—	—	1	—	15
"	F	2.5	31	6	—	2	2	—	—	—	1	—	—	15
C57BL	M	20	45	1	—	6	—	—	—	3	—	—	—	17
"	F	5	32	1	2	5	—	—	—	—	—	—	—	17
"	F	2.5	27	1	—	5	—	—	—	—	—	—	—	17
RIII	M	10	16	—	—	—	—	—	—	—	—	—	—	16
BALB/c × C3H	M	20	97	—	—	2	6	1	2	1	2	1	57	19
"	F	5	56	2	2	1	10	1	—	—	—	—	12	23
"	F	2.5	41	—	1	—	7	—	—	—	—	—	3	24
RIII × C3H	M	20	25	—	—	—	1	1	—	—	—	—	21	15
"	F	5	16	—	—	2	1	1	—	—	1	1	4	24
DBA/2 × C3H	M	20	45	1	1	2	3	5	2	—	2	—	7	21
"	F	5	39	—	—	2	3	1	—	—	2	—	2	23

"	F	2.5	29	—	—	1	1	3	—	—	1	—	1	23
I × C3H	M	20	26	—	1	1	3	4	—	1	1	—	1	20
"	F	5	20	—	2	—	4	—	—	—	1	—	1	23
"	F	2.5	15	—	2	1	1	—	—	—	—	—	—	20
C57BL × C3H	M	20	30	—	—	2	2	3	—	2	1	—	—	22
"	F	5	21	—	—	—	—	1	—	—	—	—	—	23
"	F	2.5	14	—	—	1	1	2	1	—	—	—	—	23
BALB/c × RIII	M	20	15	1	—	2	1	1	—	1	—	—	—	19
"	M	10	45	3	1	4	1	—	—	—	—	—	5	20
"	F	5	56	—	10	4	3	—	—	1	—	—	1	22
"	F	2.5	44	—	4	1	4	1	—	—	—	—	1	23
BALB/c × DBA/2	M	20	49	2	—	3	7	—	2	1	—	1	3	21
"	F	5	44	—	—	6	6	—	2	—	—	3	1	23
"	F	2.5	41	—	—	1	5	1	—	—	1	—	2	23
BALB/c × I	M	20	45	10	—	7	5	—	3	—	—	1	—	19
"	F	5	20	3	—	4	2	—	—	1	—	—	—	20
"	F	2.5	14	—	2	3	3	1	—	—	—	—	—	20
BALB/c × C57BL	M	20	35	1	1	3	8	—	—	—	—	—	—	21
"	F	5	30	—	1	3	9	—	—	—	—	—	—	22
"	F	2.5	27	—	2	1	2	1	—	—	—	1	—	22
DBA/2 × I	M	20	24	3	1	5	3	1	—	3	—	—	1	20
"	F	5	19	2	—	2	4	—	—	—	—	—	—	22
"	F	2.5	13	—	—	3	2	—	—	—	—	1	—	22
DBA/2 × C57BL	M	10	26	—	—	4	—	1	—	1	—	—	—	22
"	F	5	26	—	—	—	—	—	—	—	1	—	—	22
"	F	2.5	25	—	—	—	—	1	—	—	—	1	—	22
I × C57BL	M	20	51	4	2	6	4	1	—	8	—	—	—	19
"	F	5	29	2	1	5	1	—	—	—	—	—	—	21
"	F	2.5	23	—	—	5	2	—	1	—	—	—	—	21
Total			1633	62	41	131	144	48	19	23	22	13	152	

growths are known to occur frequently in mice and, although the experiment was planned to procure breast tumors, the number of mice with pulmonary tumors was only 8 less than those developing breast tumors. Few pulmonary tumors were prepared for microscopic examination because reliable identification of this tumor can usually be made by gross examination. Many of those found here were very large, often occupied an entire lobe, and frequently were the primary cause of death.

The results confirmed the importance of hereditary factors in the occurrence of pulmonary tumors in inbred mice (*23*). Strain BALB/c was the most susceptible and strain C57BL the most resistant. Of 659 F_1 hybrids derived from strain BALB/c, 79, or 12 percent, had pulmonary tumors, while of 245 derived from strain C57BL, exclusive of the BALB/c $\times$ C57BL hybrids, 10, or 4 percent, had this type of tumor. It is of interest that of 92 BALB/c $\times$ C57BL hybrids, 19, or 21 percent, had pulmonary tumors, the highest incidence of any group in the entire experiment. Of 104 C57BL mice and 77 DBA/2 $\times$ C57BL hybrids, none developed a pulmonary tumor.

Hepatomas are another type of tumor found in mice, occurring with greater frequency in certain inbred strains (*23*). Of the strains used in this experiment, strain C3H animals are known to be the most susceptible and, as seen in the table, this was again confirmed by autopsy findings. Strain C3H was the only inbred strain in which hepatomas were found and, of 32 found in hybrids, 23 arose in offspring of strain C3H. Very few of these neoplasms were examined microscopically because familiarity with their gross characteristics permits a diagnosis, and small tumors are not included in the table.

Nineteen mice developed tumors of the adrenal gland. All were large masses and it is safe to assume that histologic study of small nodules and enlarged adrenals would have revealed more. Hence, data in the table cannot be used to compute accurate incidences. Fifteen were diagnosed microscopically; 13 were cortical carcinomas similar to tumors previously described (*20–22*). One in an I $\times$ C57BL female hybrid had the pattern of a pheochromocytoma. One in a DBA/2 $\times$ C3H male hybrid was of unusual morphology. The cells were large and eosinophilic and resembled the cells sometimes seen in hepatomas.

The 11 males with adrenal tumors had all been castrated at an early age and their tumors probably were produced by postcastrational influences. Of the 8 females with these tumors, 4 belonged to strain BALB/c and 2 were hybrids with BALB/c parents. Of the 19 tumors, 14 arose in either BALB/c mice or their F_1 hybrids. This suggests that BALB/c animals inherit a predisposition to this type of tumor.

The observation that 23 mice had developed tumors of the pituitary gland was not unexpected because it is known that these tumors occur in mice exposed to estrogenic stimulation (*20–22*). Furthermore, 21 were males that had received 20 percent diethylstilbestrol pellets. The number of mice shown in the table is not suitable for computing tumor incidence, for only the brains of animals with signs of nervous disturbance

or intracranial pressure were examined. All were diagnosed histologically and they conformed to a common pattern (*24*). One intracranial tumor, beginning at the base of the brain and in the region of the pituitary, was found in a BALB/c × C3H hybrid female, and was diagnosed as a glioblastoma multiforme (fig. 7). We have not previously seen a tumor of this type in a mouse from our colonies.

Males of strain C57BL were the only inbred strain animals to show frank signs of pituitary tumors and this strain is known to be susceptible to estrogen-induced pituitary tumors (*20*). Analysis of 20 hybrids that had these tumors is of some interest. Eleven were procured from strain C57BL and 8 of these were of C57BL × I derivation. The presence of large tumors in 13 hybrids procured from strain I and in 6 procured from strain BALB/c suggests that these strains, or their F_1 hybrids, may also be relatively susceptible to estrogen-induced pituitary tumors. Richardson (*14*) observed high incidences of pituitary tumors in (C57L × RIII)F_1 hybrids of both sexes following implantation of diethylstilbestrol pellets.

Twenty-two mice developed sarcomas, all of which were diagnosed histologically. Eighteen were fibrosarcomas; 16 arose in subcutaneous tissues, 1 in the uterus of a DBA/2 and 1 in the uterus of a BALB/c × DBA/2 hybrid. Three were reticulum-cell sarcomas; 1 was found in the liver of a DBA/2 female, 1 subcutaneously in a BALB/c female, and 1 subcutaneously in a C57BL × C3H male. One was an osteogenic sarcoma in a strain I female.

Fibrosarcomas are known to appear spontaneously in mice of strain C3H and in their offspring (*25*). In this experiment 15 strain C3H animals or their hybrids developed sarcomas and 5 of these hybrids were derived from strain DBA/2. This, together with the 2 DBA/2 mice and 2 other DBA/2 hybrids with sarcomas, suggests that strain DBA/2 may also be relatively susceptible to the development of these tumors.

Thirteen mice developed large hemangioendotheliomas; small hemorrhagic areas were not examined microscopically. Those included in the table were confirmed microscopically. They arose in a variety of tissues and organs. In males, 2 were in subcutaneous tissues and 1 in the spleen. In females, 4 arose subcutaneously, 2 in muscle, 2 in liver, 1 in kidney, and 1 in the lining of the peritoneal cavity. It was known that BALB/c mice show a tendency to develop these tumors and the findings in this experiment supported the earlier observations. Of the 13 mice developing hemangioendotheliomas, 9 belonged to strain BALB/c or were hybrids derived from this strain.

Discussion

The primary objective of this study was attained when it was found that estrogenic stimulation can produce relatively high incidences of mammary tumors in mice of strain C3H, and in (BALB/c × C3H)F_1 and (RIII × C3H)F_1 hybrids in the absence of the mammary-tumor agent. These animals are suitable for the study of mammary tumors in agent-free mice. Intact or castrate males and nonbreeding females may be used and sufficient estrogenic stimulation can be achieved by the

simple technique of implanting diethylstilbestrol-cholesterol pellets. These pellets are easy to insert subcutaneously, are detectable by palpation, and are readily removed through a slit in the skin. They can be employed to ascertain more precisely the role of hormonal stimulation in the production of mammary tumors in mice possessing an inherited predisposition to the development of these neoplasms.

It is essential to emphasize again that the F_1 hybrids most susceptible to estrogen-induced breast tumors were derived from inbred strains most susceptible to the mammary-tumor agent. Thus, the agent accelerates the production of mammary tumors in mice possessing a tendency toward the development of these tumors when the mice are exposed to hormonal stimulation. If this generalization holds for other strains and their hybrids, then it becomes important to ascertain why those strains most susceptible to the agent are also the most susceptible to estrogen-induced neoplasms.

Although mammary tumors in the male mice receiving estrogenic hormones showed no significant morphologic variation from mammary tumors found in female mice without the agent, the histogenesis of the tumors was different. Particularly in the older male mice receiving the largest doses of diethylstilbestrol, development of the common types of tumor was usually preceded by the formation of fibrocystic tissue in focal areas of altered mammary tissue. This altered fibrocystic mammary tissue was compared with descriptions given by previous authors of mammary tissue in male mice that developed mammary tumors. It somewhat resembled: (1) a "multiple cystic state" described by Cheatle (*26*) in Lacassagne's mice, (2) "atypical growths somewhat resembling normal and functional lobules" that appeared in localized areas of the mammary duct system of 3 male mice from one strain injected with theelin by Gardner *et al.* (*27*) and, (3) descriptions by Bonser (*28*) of alterations in the mammary tissue of male mice treated with estrone. The altered fibrocystic mammary tissue in the present group of mice was often so sharply demarcated as to suggest a neoplasm. Bonser (*29*), in fact, photographed a mass composed of what appeared to be similar tissue in a RIII male mouse treated with estrogen for 65 weeks. She named the lesion "cystic adenoma," an appropriate designation in our opinion. Foulds (*30*) also described a preneoplastic hormonally dependent lesion or "plaque." Although the plaque described by Foulds differed morphologically from the fibrocystic tissue observed by us, the lesions had in common the capacity of progression, and the usual tumor types developed within them. Histogenesis of the tumors in the male mice of the present experiment differed from the histogenesis of mammary tumors in strain C3H mice with the agent, where a preneoplastic lesion designated a "hyperplastic nodule" and composed of small acinar-like tissue (*18*) preceded the tumor development. Similar nodules were also described in hybrid mice theoretically free of the agent (*19*).

When these different modes of development for mammary tumors in mice are considered, it is evident that the histogenesis of the tumors

presents the same diversity as was found in the morphologic patterns of the tumors themselves, and that the modes of origin are probably no more uniform than are the resultant tumors. These differences in histogenesis have been related to the inbred strain, the age, the sex, the presence of the agent, and hormone treatment. All these factors should be considered when either the histogenesis or the morphology of mammary tumors is described, and it is not surprising that different investigators have observed different modes of origin of mammary tumors in mice.

The fibrocystic lesion described herein may have been hormonally dependent and reversible in the early stages of development, but the experiments were not designed to investigate this point. As was to be expected, hormonally induced tumors besides the mammary tumors were found in these estrogen-treated mice. A number of adrenocortical tumors and pituitary tumors appeared. In general, the incidence of other tumor types was related to genetic factors. Estrogenic hormones appeared to increase the number of reticular neoplasms.

Although glioblastoma multiforme has been reported in mice following the intracerebral instillation of carcinogens (*31*, *32*), we have not found a previous report or observed such a neoplasm in an intact mouse.

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PLATES

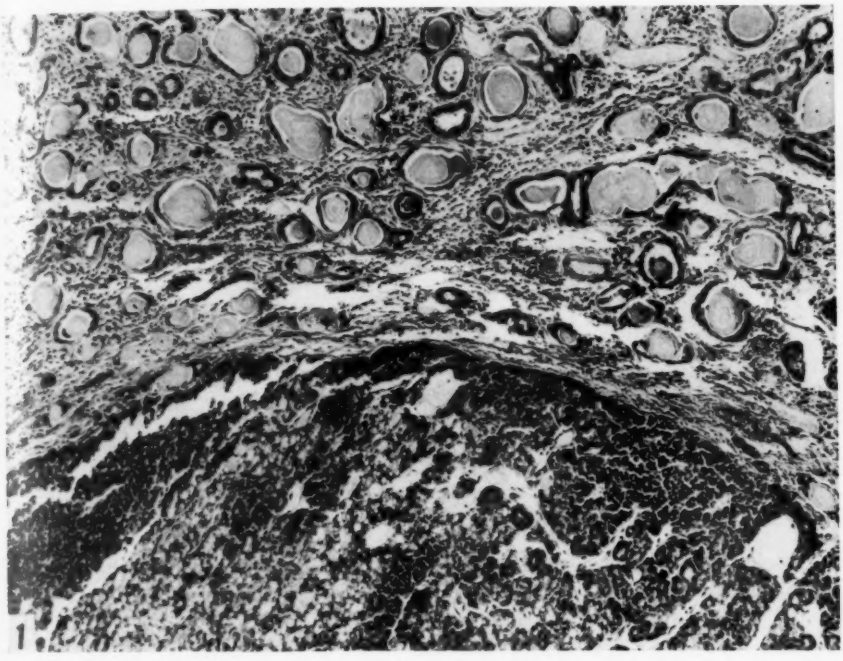
PLATE 78

All sections stained with hematoxylin and eosin.

FIGURE 1.—(BALB/c × C3H+)F_1 hybrid castrate male, 14 months old, received a 20 percent diethylstilbestrol pellet. Type A adenocarcinoma *below* and fibrocystic tissue *above*. The fibrocystic tissue is composed of small cysts lined by cuboidal and flat epithelial cells, and contains concentric, eosinophilic bodies. The cysts are separated from each other by thick, collagenous connective tissue. The tumor has a central area containing many vacuoles, indicating secretory activity. × 90

FIGURE 2.—(BALB/c × C3H)F_1 hybrid castrate male, 14 months old, received a 20 percent diethylstilbestrol pellet. A mass of fibrocystic tissue containing two adenocarcinomas, type B. The margins of the fibrocystic lesions were sharply defined. *Note* compression of fibrocystic tissue by expanding tumors. × 8

FIGURE 3.—(BALB/c × C3H)F_1 hybrid castrate male, 12 months old, received a 20 percent diethylstilbestrol pellet. Mass of fibrocystic tissue with adenocarcinoma, type B, *center*. × 8



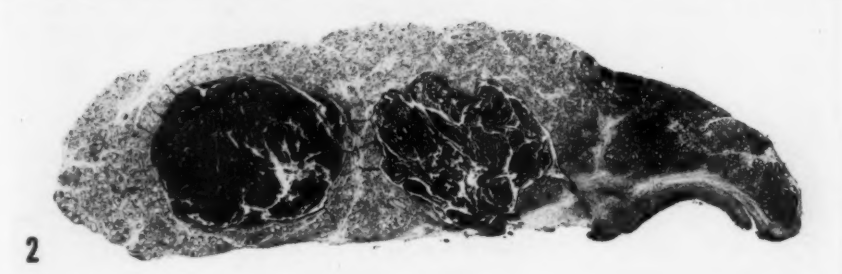


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PLATE 79

FIGURE 4.—(BALB/c × C3H)F_1 hybrid castrate male, 16 months old, received a 20 percent diethylstilbestrol pellet. Adenocarcinoma, type A *below*, fibrocystic mass *at left* and estrogen-stimulated mammary tissue *upper right*. Epithelial lining cells in some of the cysts show proliferation and papillary ingrowth. × 60

FIGURE 5.—(BALB/c × C3H+)F_1 hybrid castrate male, 12 months old, received a 20 percent diethylstilbestrol pellet. A small focus of adenocarcinoma within a fibrocystic mass. × 60

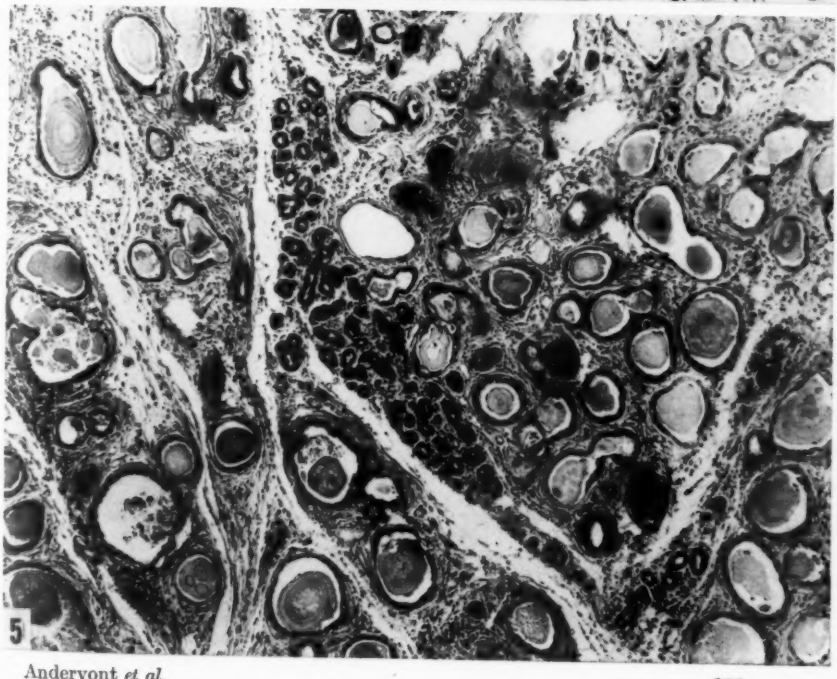

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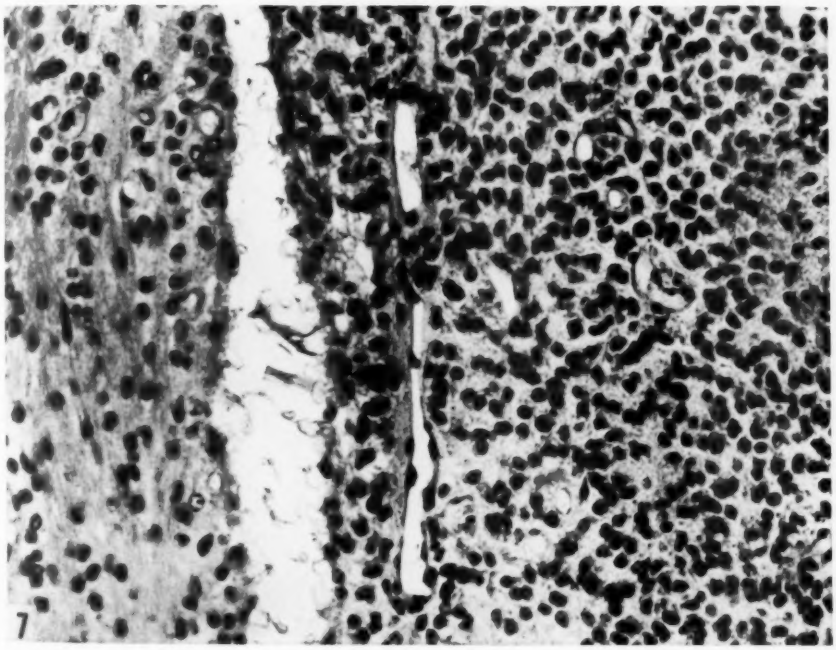
PLATE 80

FIGURE 6.—(BALB/c × C3H)F_1 hybrid castrate male, 17.5 months old, received a 20 percent diethylstilbestrol pellet. Fibrocystic tissue within and around inguinal lymph node indicates neoplastic nature of this lesion as judged by its ability to infiltrate lymph node. × 60

FIGURE 7.—(BALB/c × C3H)F_1 hybrid female, 20 months old, received a 5 percent diethylstilbestrol pellet. Glioblastoma multiforme. Normal brain tissue *on left*, cellular glioblastoma *on right*, separated by ventricular space. × 290



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